REMARKS

FORMAL MATTERS:

Claims 1-10 and 12-15, 20-27, and 40-49 are pending after entry of the amendments set forth herein.

Claim 11 is canceled without prejudice.

Claims 16-19 and 28-39 are canceled without prejudice as being drawn to non-elected subject matter.

Claims 1, 5, 7-9, 14, and 21-22 are amended for further clarity and/or to correctly typographical errors.

New claims 40-49 are added. Support for these new claims is found in, for example, original claim 1-8; and specification paragraph [0084].

The specification is amended to properly refer to the trademarks kindly noted by the Examiner.

Applicants note the several claims were previously amended in a Preliminary Amendment mailed January 16, 2002.

No new matter is added.

OVERVIEW OF THE RESPONSE

The present invention is directed to a method for eliciting protective immunity against *Neisseria meningitidis* by administration of preparations of microvesicles (MV), outer membrane vesicles (OMV), or both MV and OMV from *N. meningitidis* as recited in the claims.

Rejections under §112, ¶2

The claims were variously rejected under §112, ¶2. These rejections are addressed herein.

Rejections under §112, ¶1

The claims were also rejected under §112, ¶1 on the grounds that the specification, while enabling for methods of producing neutralizing antibodies, does not provide an enabling disclosure for methods directed to eliciting broad spectrum protective immunity against *Neisseria meningitidis* and diseases caused by *N. meningitidis* species. Applicants have shown that the claimed methods elicit bactericidal antibodies in mice and in guinea pigs, which bactericidal antibodies provide for passive

protection against bacterial challenge in an infant rat model. However, the Examiner indicated during the interview that while the data in the specification was extensive, the specification did not provide data using an animal model in which both immunization and challenge occurred.

As discussed during the interview, *N. meningitidis* is unique with respect to immune response sufficient to provide protective immunity. Unlike many other pathogenic organisms, the presence of bactericidal antibodies is an indicator of protective immunity. Protective immunity and bactericidal antibodies go hand-in-hand. This is not to imply that bactericidal antibodies are the only measure of a protective immune response, or are necessarily or absolutely required. However, if bactericidal antibodies *are* in fact present, it is well-accepted that protective immunity is present. The presence of bactericidal antibodies is so well-established as an indicator of protective immunity that a measure of bactericidal antibodies has been a primary basis for licensure of *N. meningitidis* vaccines without the need for an efficacy trial. As discussed in more detail below, this has been true for both conjugate vaccines and outer membrane vesicle (OMV) vaccines.

The specification has shown that the claimed method results in production of bactericidal antibodies, which elicit activity in both *in vitro* assays as well as in an *in vivo* animal model. Applicants respectfully submit that the specification is fully enabling and this rejection can be withdrawn. Applicants' position in this regard is set forth in more detail below.

INTERVIEW SUMMARY

Applicants wish to express their gratitude to Examiner Zeman for the telephonic interview on May 13, 2004. The undersigned and the co-inventors Dan Granoff and Gregg Moe participated.

The rejections of the claims under §112, ¶2 and §112, ¶1 were discussed. Specifically, the Examiner agreed that the amendments and remarks as set out herein would serve to obviate the rejections under §112, ¶2. In addition, the Examiner indicated he would consider favorable applicants' arguments regarding the enablement rejection under §112, ¶1 in view of the assertions regarding the role of serum bactericidal antibodies in providing protective immunity against *Neisseria meningitidis*, as discussed in detail below.

OBJECTIONS TO THE SPECIFICATION

Use of trademarks

The objection to the specification as it relates to the use of trademarks is addressed by amendment, and may be withdrawn.

ATCC deposit numbers

With respect to the objection to the specification relating to incomplete ATCC deposit information, applicants note that paragraph [00207] on page 60 of the specification was amended to include the deposition information in Preliminary Amendment, filed August 21, 2001.

Page 51 does not refer to any specific deposit information that is missing. The prior amendment to page 61 in the Preliminary Amendment should be sufficient to address this informality.

Withdrawal of the objections to the specification is respectfully requested.

OBJECTIONS TO THE CLAIMS

The objection to claim 11 is rendered moot by cancellation of this claim. Withdrawal of this objection is respectfully requested.

REJECTIONS UNDER §112, ¶1

Claims 1-15 and 20-27 were rejected on the grounds that, while enabled for methods of producing neutralizing antibodies, the claims methods are not enabled for eliciting broad spectrum protective immunity against *Neisseria meningitidis* and diseases caused by *N. meningitidis* species.

The Office Action bases this rejection on the assertion that the specification does not set forth that the claimed invention provides any sort of protective immunity in any model system that can be extrapolated to humans or other mammals. (Office Action, page 3, bottom) The Office Action states that

> Applicant describes methods of producing antibodies (i.e. antisera) that is [sic] bactericidal *in vitro* but fails to demonstrate that the claimed method provides protective immunity (broad spectrum or otherwise) in any animal system other than ruminants.

Moreover, the specification is silent on what 'diseases' can be prevented by the claimed methods." (Office Action, text bridging pages 3-4)

This rejection is respectfully traversed.

First, with respect to the aspect of the rejection relating to the "diseases" that can be prevented by the claimed methods, applicants respectfully submit that the amendments to the claims serve to further clarify the claimed invention, rendering this rejection moot.

The remaining aspects of the rejection are addressed below.

The specification provides ample evidence to support the assertion that the claimed methods provide to protective immunity against multiple strains, including serotype and serosubtypes, of N. meningitidis

As stated in the specification at paragraph [00178], finding that a vaccine produces bactericidal antibodies against *Neisseria meningitidis* is accepted in the field as an indicator of the vaccine's protective effect.[1] This is true of both conjugate-based[2], outer membrane protein-based and conjugate-based,[3] and outer membrane vesicle(OMV)-based[4] vaccines. As also stated in the specification at paragraph [00184], the infant rat model is a model of *N. meningitidis* infection.

The specification provides data showing that the claimed invention provides for production of anti-*N. meningitidis* antibodies that are bactericidal (specification paragraphs [00178] – [00183]) and that provide for passive protection against infection in the infant rat model (specification paragraphs [00184] – [00187]).

While applicants believe the statements above should suffice to overcome this rejection, for the Examiner convenience, the discussion below provides more details in support of applicants' position. Applicants provide 1) a review of the literature supporting the assertion that production of bactericidal

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[1] See, e.g., Goldschneider et al., Human immunity of the meningococcus, I. The role of humoral antibodies, 1969, *J. Exp. Med.* 129:1307 (Exhibit 1).
[2] Borrow et al., Serological basis for use of meningococcal serogroup C conjugate vaccines in the united kingdom: reevaluation of correlates of protection, 2001 Infect Immun. 69:1568 (Exhibit 2).
[3] Milagres et al., Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect Immun 1994;62:4419-24 (Exhibit 3)
[4] Holst et al., Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease. Vaccine 2003, 21:734-737 (Exhibit 4).

antibodies is evidence suggesting that a protective immune response has been elicited; and 2) a discussion of the results in animal models as presented in the specification.

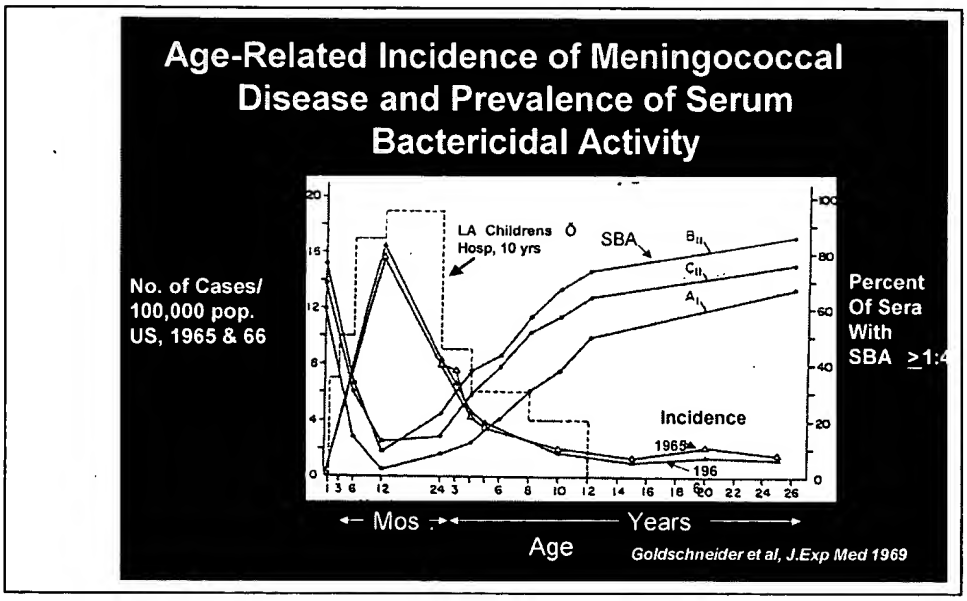
Bactericidal activity of antibodies against Neisseria meningitidis is an art-accepted indicator of predictor of protection against invasive meningococcal disease

The bactericidal assay measures the interaction of antibody and complement at the bacterial surface, which results in bacterial death. Several lines of evidence, summarized below, prove that the presence of serum bactericidal activity is a reliable predictor of protection against invasive meningococcal disease (disease defined as invasion of the bloodstream by the bacteria [bacteremia] and/or invasion of the membranes covering the brain (meningitis).

1. Age-incidence of meningococcal disease is inversely related to the prevalence of meningococcal disease

Most newborns have serum bactericidal antibodies that are acquired transplacentally from their mothers and persist in the infant for a few months (see graph below).[5] Thereafter, natural acquisition of serum bactericidal antibodies to *N. meningitidis* is inversely related to age. Low levels of bactericidal antibodies are present below 2 years, which corresponds to the age group at greatest risk of acquiring meningococcal disease. Between 2 and 12 years of age there is a progressive increase in the prevalence of serum bactericidal antibody, which coincides with a progressive decrease in the incidence of meningococcal disease in the population.

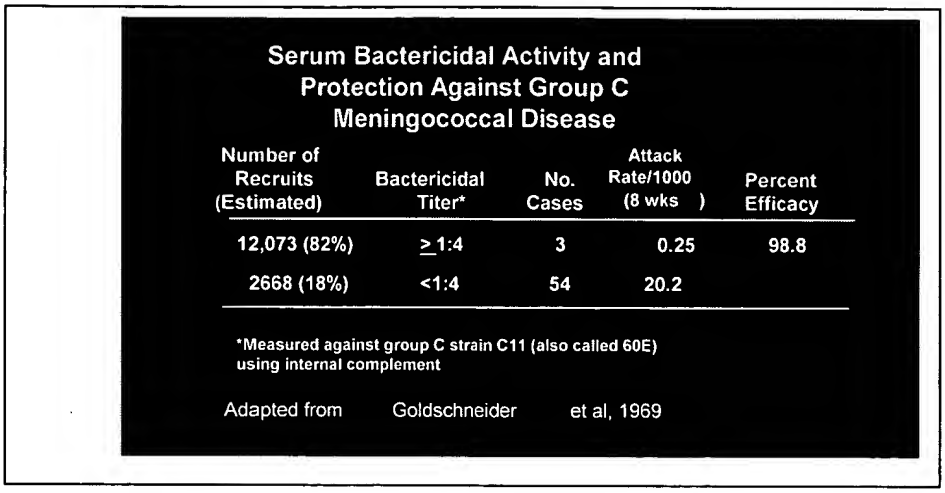
[5] Goldschneider et al., *supra* (Exhibit 1)



2. Persons with naturally-acquired serum bactericidal antibody are protected against developing meningococcal disease.

In their seminal study, Goldschneider et al demonstrated the importance of naturally-acquired serum bactericidal antibodies in protection against group C meningococcal disease during an epidemic among military recruits in the 1960s.[6] Group C bactericidal antibodies were present in baseline sera of approximately 82 percent of the recruits. Those with detectable serum bactericidal antibody frequently became carriers of the epidemic strain but did not develop meningococcal disease while virtually all cases of disease occurred in the 18 percent of individuals whose baseline sera lacked bactericidal activity (titers <1:4 measured with human complement). Recruits who lacked serum bactericidal antibody and developed group C carriage had meningococcal disease attack rates as high as 38.5 percent. The "efficacy" of a bactericidal titer of 1:4 or greater was >98%.

[6] Goldschneider et al., *supra* (Exhibit 1).



Serum Bactericidal Activity and Protection Against Group C Meningococcal Disease

Number of Recruits (Estimated)	Bactericidal Titer*	No. Cases	Attack Rate/1000 (8 wks)	Percent Efficacy
12,073 (82%)	≥1:4	3	0.25	98.8
2668 (18%)	<1:4	54	20.2	

*Measured against group C strain C11 (also called 60E) using internal complement

Adapted from Goldschneider et al, 1969

3. Serum bactericidal activity is accepted in the art as a correlate with vaccine efficacy against Neisseria meningitidis

As a result of the seminal Goldschneider data, serum bactericidal antibody (SBA) titers are a principal basis for licensure of meningococcal vaccines.[7,8,9,10]

Notably, the World Health Organization,[11] and regulatory agencies in Europe and the U.S., require a showing of serum bactericidal antibody responses as an indicator of vaccine efficacy for licensure of new meningococcal vaccines. For licensure of the tetravalent meningococcal polysaccharide vaccines in the U.S, the efficacy of the group Y and W135 components was inferred based on 4-fold or greater bactericidal antibody responses.[12] In the United Kingdom, the efficacy data supporting licensure of the new group C meningococcal polysaccharide-protein conjugate vaccines were based on serologic responses, with bactericidal titers being the most important single criterion.[13] These vaccines were subsequently licensed in Europe, Canada and elsewhere based on these data.

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[7] Borrow et al., *supra* (Exhibit 2).
[8] Balmer, et al., Serologic correlates of protection for evaluating the response to meningococcal vaccines. Expert Rev Vaccines 2004;3:89-99 (Exhibit 5).
[9] Milagres et al., *supra* (Exhibit 3)
[10] Holst et al., *supra* (Exhibit 4)
[11] World Health Organization. Requirements for meningococcal polysaccharide vaccine (requirements for biological substances no. 23). WHO Tech Rep Ser 1976;594:1-86, see particularly pages 19 and 72 – 73 (Exhibit 6)
[12] 50 Fed. Reg. 162, Guidelines for Production of Meningococal Polysaccharide Vaccines Docket No. 84D-0263, Notice of Availability Published August 21, 1985 (Exhibit 7).
[13] Borrow et al., *supra* (Exhibit 2)

4. The application provides evidence of production of bactericidal antibodies in mice and in guinea pigs following vaccination according to the claimed methods

The specification provides data showing that the claimed invention provides for production of anti-*N. meningitidis* antibodies that are bactericidal not only against strains of the same serotype or serosubtype as the strains used to generate the vesicles used in vaccination, but also against strains that were of a different serotype or serosubtype (specification paragraphs [00178] – [00183]). The production of such bactericidal antibodies was shown in both mice and in guinea pigs. Immune responses in mice in guinea pigs can differ from those in humans; however, the ordinarily skilled artisan would find this data in two different animal models sufficient evidence to merit the inordinate expense involved in trials in a primate model or in humans.

In short, this data provides an indication that bactericidal antibodies can indeed be produced according to the claimed method. The mice and guinea pigs developed high serum titers of bactericidal antibody, which in humans is the accepted correlate of protection against meningococcal disease.

Passive protective activity in the infant rat model.

The inventors described in the application an infant rat meningococcal bacteremia model, which can be used for measuring antibody protective activity against group B or C strains.[14],[15] This model permits investigation of the protective activity of antibodies in a setting where the organism is rapidly replicating *in vivo*.

The specification at paragraphs [00-184] – [00187] and in Figure 10 provides direct data proving that the antibodies elicited in mice by the claimed vaccination method passively confer protection against meningococcal bacteremia in the infant rat model. Figure 11 shows similar data showing that antibodies elicited in guinea pigs by the claimed method also provide for protecting against meningococcal bacteremia in the infant rat model. Thus, applicants have shown, in two different animal species, that the claimed vaccination method elicited antibodies that confer passive protection against meningococcal bacteremia in a bacteremia model. Thus, the bactericidal effect of the serum antibodies

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[14] Moe et al., Differences in surface expression of NspA among Neisseria meningitidis group B strains. Infect Immun 1999 67:5664-75 (Exhibit 8).

[15] Harris et al., Age-related disparity in functional activities of human group C serum anticapsular antibodies elicited by meningococcal polysaccharide vaccine. Infect Immun 2003;71:275-86 (Exhibit 9).

produced by the method are not only effective in an in vitro assay, but are also effective in vivo in an animal model of bacteremia.

Conclusion

In view of the ample data in the application relating to production of bactericidal antibodies, and further in view of the additional data from the infant rat model experiments, applicants respectfully submit that the specification provides an enabling disclosure as required by §112, ¶1.

Withdrawal of this rejection of the claims is respectfully requested.

REJECTIONS UNDER §112, ¶2

Claims 1-8 and 20-27 were variously rejected under §112, ¶2. These rejections are specified and addressed below:

Claim 1

Claim 1 was rejected for recitation of "third species" and "third preparation". This rejection is rendered moot by amendment.

Claims 1 and 14, and claims 7 and 9

Claims 1 and 14 were rejected for recitation of "a disease caused by more than one strain of *Neisseria meningitidis* species". Claims 1, 14, 7 and 19 – as well as claim 8 – are amended for clarification to further clarify the invention.

Withdrawal of this rejection is respectfully requested.

Claim 21

Claim 21 was rejected for recitation of "excipients" without antecedent basis. This rejection is rendered moot by amendment, and may be withdrawn.

Claim 22

Claim 22 was rejected for recitation of "the adjuvant" without antecedent basis, and for recitation of improper Markush language. This rejection is rendered moot by amendment, and may be withdrawn.

Withdrawal of the various rejections of the claims under §112, ¶2 is respectfully requested.

INFORMATION DISCLOSURE STATEMENT:

The Applicants note that an Information Disclosure Statement (IDS), including an SB/08A form, was submitted in this application on April 22, 2002. The Applicants respectfully request that the Examiner initial and return this SB/08A form, thereby indicating that the references cited in the IDS have been reviewed and made of record. For the Examiners convenience, a copy of this form is enclosed herewith.

Applicants also submitted a further IDS with the originally filed Amendment on June 21, 2004, including an SB/08A form, along with copies of the references cited therein. Applicants respectfully request that the Examiner initial and return the SB/08A form, thereby indicating that the references cited in the IDS have been reviewed and made of record.

Applicants note that the PAIR page for the application indicates that the IDS initially submitted with the Amendment filed on June 21, 2004, had been entered. Accordingly, Applicants have not re-submitted the IDS with this communication.

CONCLUSION

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number CHOR-001.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: Sept 14, 2004

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Enclosures: Exhibits 1-9

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Exhibit 1

HUMAN IMMUNITY TO THE MENINGOCOCCUS

I. The Role of Humoral Antibodies

II. Development of Natural Immunity

By IRVING GOLDSCHNEIDER, M.D., EMIL C. GOTSCHLICH, M.D., and MALCOLM S. ARTENSTEIN, M.D.

III. Preparation and Immunochemical Properties of the Group A, Group B, and Group C Meningococcal Polysaccharides

By EMIL C. GOTSCHLICH, M.D., TEH YUNG LIU, Ph. D., and MALCOLM S. ARTENSTEIN, M.D.

IV. Immunogenicity of Group A and Group C Meningococcal Polysaccharides in Human Volunteers

V. The Effect of Immunization with Meningococcal Group C Polysaccharide on the Carrier State

By EMIL C. GOTSCHLICH, M.D., IRVING GOLDSCHNEIDER, M.D., and MALCOLM S. ARTENSTEIN, M.D.

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HUMAN IMMUNITY TO THE MENINGOCOCCUS

I. The Role of Humoral Antibodies

By IRVING GOLDSCHNEIDER, M.D., EMIL C. GOTSCHLICH, M.D., and MALCOLM S. ARTENSTEIN, M.D.

(From The Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D. C. 20012)

(Received for publication 20 February 1969)

Meningococcemia and meningococcal meningitis in man appear to be infrequent complications of the host-parasite relationship. Ordinarily, exposure to a strain of *Neisseria meningitidis* results in carriage of the organism in the nasopharynx for weeks or months (1–4), producing either a mild pharyngitis or no symptoms at all (5, 6). Even during an epidemic, the great majority of individuals exposed to the epidemic strain of meningococcus become asymptomatic carriers rather than clinical cases with systemic disease (7). Viewed in this light, the occurrence of meningococcal disease is related more to the unique susceptibility of the individual host than to the innate virulence of the infecting organism.

Several epidemiological findings suggest that individuals who are susceptible to systemic meningococcal disease lack humoral antibodies to meningococci. First, except for outbreaks among military recruits (8, 9) and other closed populations (10), meningococcal meningitis is a disease of infancy and early childhood (11, 12). This is true both in epidemic and interepidemic periods. Susceptibility to many so-called "diseases of childhood," bacterial and viral, has been shown to be associated with deficient levels of circulating antibodies to the infecting microbe (13). Second, the cyclical occurrence of epidemic meningococcal disease in the United States at approximately 10 yr intervals (14) suggests that the disease depends for its expression on the presence of an immunologically virgin population. Third, meningococcemia seems to confer permanent immunity to future attacks of systemic meningococcal disease, although this is difficult to test statistically (10, 15). Convalescent sera from such cases have elevated levels of precipitating, agglutinating, complement-fixing, opsonizing, bactericidal, and mouse-protective antibodies to the infecting meningococcus (16–18).

The absence of a suitable animal model for the experimental study of meningococcemia and meningococcal meningitis has impeded the evaluation of the host-parasite relationship. Antibodies to meningococci have been found to be present normally in blood from mouse, rat, guinea pig, rabbit, rhesus monkey, chimpanzee, horse, and ox (19).[1] It is possible that these antibodies are responsi-

[1] Goldschneider, I. Unpublished observations.

ble, at least in part, for the insensitivity of such animals to meningococcal disease. Similarly, antimeningococcal antibodies have been found in normal human blood (19–22). These observations, together with the epidemiological findings presented above, suggest that humoral antibodies may be important in preventing systemic meningococcal disease in man.

Such an idea is not new. Kolmer et al. (23) showed that many normal human sera contain opsonic antibodies to meningococci isolated from cases of meningitis, and postulated that such antibodies might be protective. Heist et al. (24) found that blood from more than 95% of normal men was bactericidal to meningococcal strains obtained from asymptomatic carriers. They postulated that most cases of meningitis occur among those few individuals who lack bactericidal activity. Dr. Heist, whose own blood was nonreactive against meningococci, died with meningococcal meningitis. While these early experiments were suggestive of a protective role of circulating antibody against meningococcal disease, definitive evidence was lacking. The presentation of such evidence is the subject of this paper.

Methods

Bacteriological Techniques.—

Bacteria: Strains of *Neisseria meningitidis* were obtained from the collection of the Department of Bacteriology, Walter Reed Army Institute of Research, and from surveys conducted at several military installations, as indicated in the text. Specimens from blood and cerebrospinal fluid were plated on "chocolate" agar (Mueller-Hinton base [25], Difco Laboratories, Detroit, Mich.), and those from nasopharynx on "chocolate" agar containing 6 μg lincomycin hydrochloride monohydrate (Upjohn Co., Kalamazoo, Mich.) and 25 units polymyxin B sulfate (Charles Pfizer & Co., New York) per milliliter (26). The plates were incubated for 16 to 24 hr at 37°C in the presence of moisture and CO_2 (candle jar). Bacterial isolates were subcultured once on chocolate agar, without antibiotics, and identified as meningococci by serogrouping and fermentation of carbohydrates. The strains were preserved by lyophilization, and by freezing in a preserving solution at −70°C. The preserving medium (27) consisted of 5% (w/v) bovine serum albumin (Calbiochem, Los Angeles, Calif.) and 5% (w/v) monosodium glutamate (Sigma Chemical Co., St. Louis, Mo.) in distilled water. Renewal of the working (frozen) stock was made from the lyophile collection. Thus, all meningococcal strains used in the present experiments were within two passages of original isolation.

Serogrouping: The slide agglutination technique (28) was employed for routine serogrouping of meningococci. Commercially obtained typing sera (Difco) were used to identify group A, B, and C organisms; and rabbit antisera prepared in this laboratory against prototype organisms were used to type group Boshard (Bo), 135, and 29-Eur (29), and group X and Z (30). Serogroups Bo and Y (30) were considered to be identical (29). Meningococci which agglutinated spontaneously in saline, which were agglutinated by antisera to several serogroups, or which were not agglutinated by any serum in the panel were called "nontypable" (NT).

Carbohydrate fermentation: The bacterial isolates were tested for carbohydrate fermentation by a replicate plating method using a modified Lidwell phage applicator (31). Each strain was tested against dextrose, maltose, sucrose, and lactose. Only bacteria which had typical colonial morphology and fermented dextrose and maltose, or dextrose alone in the case of some sulfonamide-resistant organisms (32), were classified as *N. meningitidis*.

Preparation of bacterial suspensions: Bacteria were cultured on "chocolate" agar (Mueller-Hinton base, Difco lot No. 496202) for 16 hr at 37°C in the presence of moisture and CO_2 (candle jar). The organisms were subcultured for 5 hr on fresh chocolate agar and suspended in Dulbecco's (33) phosphate-buffered saline (PBS) pH 7.2 (Grand Island Biological Co. Grand Island, New York). The concentration was adjusted to an optical density of 0.20 at

TABLE I

Sources of Children's Sera

Group No.	Location	Donors		
		Age range	Clinical history	No. sera
1	Beth Israel Hosp., Boston (Dr. Ronald Gold)	Newborn	Normal	12
2	Univ. of Miami, Florida (Dr. Bernard Fogel)	Newborn—6 months	Normal	45
3	Georgetown Univ., Washington, D. C. (Dr. Joseph Bellanti)	6 months–2 yr	Recurrent upper respiratory infections	16
4	Walter Reed Gen. Hosp., Washington, D. C. (Dr. Wm. Stewart)	6 months–12 yr	Normal	45
5	Hosp. for Sick Children, Washington, D. C. (Dr. Joseph Bellanti)	6 months–12 yr	Chronic noninfectious illnesses	48
6	Junior Village, Washington, D. C. (Dr. Albert Kapikian)	6 months–12 yr	Normal	79
7	Div. Biol. Standards, N. I. H., Bethesda, Md. (Dr. Paul Parkman)	7 months–12 yr	Normal	37
Total....				282

650 mμ (16 × 125 mm Pyrex screw cap tube, Meteor Glass Co., Vineland, N. J.) in a spectrophotometer (Coleman Jr., model 6A). This corresponded to a colony count of approximately 10^9 bacteria/ml. Appropriate dilutions were made in PBS. It was found that the total particle count, as measured in a hemocytometer, approximated (±10%) the viability count (colony count).

Sera.—Human blood was collected by venipuncture, allowed to clot for 60 min at room temperature, and refrigerated at 4°C for 3 hr. After centrifugation (1500 *g* for 10 min) the sera were placed in sterile 5 or 9 ml screw cap vials and stored at −70°C. Sera were not refrozen more than three times. $C'H_{50}$ hemolytic units (34) were determined on all sera defrosted more than once.

Children: Table I shows the sources of sera from children. None of the children were receiving antibiotics, gamma globulin, or immunosuppressant drugs.

Adults: Serum was obtained from army recruits (ages 19–26 yr) during their 1st wk of basic training at Fort Dix, N. J., 1966–1968. Blood was drawn prior to the routine post-induction immunizations.

Gamma Globulin.—Lyophilized, pooled human gamma globulin (Cohn fraction II, Lot No. 2191) was obtained from E. R. Squibb, Inc., New Brunswick, N. J. The gamma globulin was dissolved in PBS at a concentration of 10% (w/v) and centrifuged at 105,000 *g* (Spinco ultracentrifuge, model L) for 5 hr. The upper two-thirds of the supernate was retained, stored at 4°C, and used within 24 hr of preparation.

Complement.—The source of complement was normal human serum (P. M.) which lacked bactericidal activity to the strains of *N. meningitidis* used in this study. The serum was divided into aliquots, stored at −70°C, defrosted immediately prior to use and not refrozen. The donor was cultured repeatedly to ascertain that he did not become a carrier of meningococci during the study.

In all instances in which exogenous complement was *not* added to the bactericidal system $C'H_{50}$ units/ml were determined by the method of Hook and Muschel (34). Only sera having 140 or more $C'H_{50}$ units/ml were considered in tabulating results. Amboceptor-coated sheep red blood cells were kindly provided by Mr. Earl Fife, Department of Serology, Walter Reed Army Institute of Research.

Antibody Assays.—

1. Serum bactericidal reaction: The serum bactericidal test was performed in a Microtiter system (35) using disposable U-well trays (Linbro Chemical Co., New Haven, Conn.), and 25 and 50 μl droppers and 25 and 50 μl diluters (Cooke Engineering Co., Alexandria, Va.). Plastic materials were sterilized by exposure to ethylene oxide (Anprolene, C. R. Bard, Inc.) for 16 hr. The diluters were flamed and allowed to cool prior to use.

The reaction mixture had a total volume of 0.2 ml and consisted of one part diluted serum (or gamma globulin), one part PBS, one part complement (or PBS), and one part bacterial suspension (approximately 10^4 bacteria/ml), added in that order. Dulbecco's phosphate-buffered saline (10^{-3} M Ca^{++}, 5×10^{-4} M Mg^{++}) was the diluent. Complement and heat-inactivated serum controls (56°C for 30 min) were included in each experiment.

The tray was sealed with sterile (UV irradiation) transparent tape (Cooke Engineering Co.), forcefully inverted to mix the reactants, and incubated at 37°C for 30 min. After removing the seal, the Microtiter tray was placed on edge and supported at an angle of approximately 75° to horizontal. Fluid from each well was allowed to flow into a glass capillary (Fisher Scientific Co., Pittsburgh, Pa., No. 12-141), and a drop was deposited on Mueller-Hinton agar which had been partially dehydrated by storage at room temperature for 4 to 8 days. Rapid absorption of fluid by the agar terminated any residual bactericidal activity and also prevented dispersion of dividing bacteria. The capillaries delivered approximately 0.02 ml per drop. In the absence of bactericidal activity such a drop contained approximately 50 colony-forming units. The inoculated plates were incubated at 37°C for 18 to 24 hr and colony counts performed.

a. Interpretation of bactericidal test: Replicate colony counts in several hundred experiments showed the error in the system to be ±20%. For this reason, only colony counts which were less than 50% of control levels were considered indicative of bactericidal activity. The 50% level of killing was also chosen to maximize the sensitivity of the bactericidal assay.

The 30 min incubation period for the bactericidal reaction was selected for three reasons. First, sequential colony counts showed that the serum bactericidal activity against meningococci was completed by 30 min (usually by 15 min). Second, meningococci placed in lag phase by suspending in PBS at room temperature did not divide in the complement or heated-serum controls during the 30 min incubation period, but did so after 60 min. Third, viability counts

of meningococci in PBS (or high serum dilutions) remained constant for 60 min, but spontaneously decreased thereafter.

Agglutination of bacteria did not present a problem in the interpretation of colony counts after exposure of meningococci to serum. In low titer sera (bactericidal activity in dilutions less than 1:32) there was no significant agglutination. Such sera, when decomplemented by heating at 56°C for 30 min, did not produce any depression in colony counts compared to complement or PBS controls. However, antibodies were apparently unaffected by the heat inactivation as evidenced by restoration of bactericidal activity after addition of complement, and by continued binding of specific IgG, IgM, and IgA antibodies to meningococci as determined by indirect immunofluorescence (*vide infra*). Agglutination *did* occur in some high titer sera, but was of no practical consequence in the interpretation of bactericidal activity. With few exceptions, there was an abrupt transition between serum dilutions producing 100% killing and those showing 0% killing.

Consistent results in the serum bactericidal system were obtained if there was strict adherence to the procedures outlined. However, deviation in age of the bacterial culture (less than 4 hr or more than 6 hr), use of a different lot number of Mueller-Hinton agar, or repeated passage of the meningococcal strain in vitro affected the reproducibility of the system, (i.e., increased or decreased sensitivity of the meningococcal strain to the lethal action of antibody and complement). For this reason, comparable experiments were done at one time, or if this was not possible, representative specimens were included as controls in subsequent experiments.

2. Immunofluorescence: Indirect immunofluorescence was done using standard techniques (36). The antigens were 5 hr cultures of meningococci, washed once with distilled water, suspended in distilled water at a concentration of 2×10^8 bacteria/ml, and dried onto slides at 37°C. Human sera were used in a standard dilution of 1:2 or titrated by serial 2-fold dilutions. Fluorescein-conjugated rabbit antisera to heavy chains of human IgG, IgM, or IgA globulins were used in a 1:20 dilution (Behringwerke, Marburg-Lahn, Germany; distributed by Hoechst Pharmaceutical Co., Kansas City Mo.). Phosphate-buffered saline, pH 7.2 was the diluent throughout.

The antigen was incubated at room temperature with human serum for 20 min, washed in three changes of PBS (5 min each), reincubated for 20 min in fluorescein-conjugated rabbit antiserum, rewashed, and mounted in buffered glycerin (Difco). Specimens were examined in a Zeiss Standard Universal microscope (Carl Zeiss, Inc., Oberkochen, West Germany), equipped with a 100 × apochromatic oil immersion objective with iris diaphragm, 8 × eyepiece and 1.25 × Optovar magnifying attachment. The ultraviolet light source was a 200 w mercury vapor arc lamp. A BG 12 excitation filter and a combination of barrier filters Nos. 53 and 44 were used.

The sensitivities of the bactericidal and immunofluorescence (anti-IgG) assays for the detection of antimeningococcal antibodies in human sera were similar (± one 2-fold serum dilution).

RESULTS

Age-Related Immunity—Fig. 1 shows the relationship between serum bactericidal activity against *N. meningitidis* and the incidence of systemic meningococcal disease in the general population, newborn to 26 yr of age. Three strains of meningococci, A1 (serogroup A), B11 (serogroup B) and C11 (serogroup C) from cases of meningitis were chosen as prototypes. These are the serogroups responsible for over 90% of cases of meningococcal meningitis (37). Age-specific morbidity rates for meningococcal infections in the United States in 1965 and

1966 were obtained from the National Communicable Disease Center, Atlanta, Ga.[2] Attack rates were calculated using estimated population bases for the various age groups (38). In addition, a survey of the age distribution of 72 cases of meningococcal meningitis admitted to Los Angeles Children's Hospital (1944–

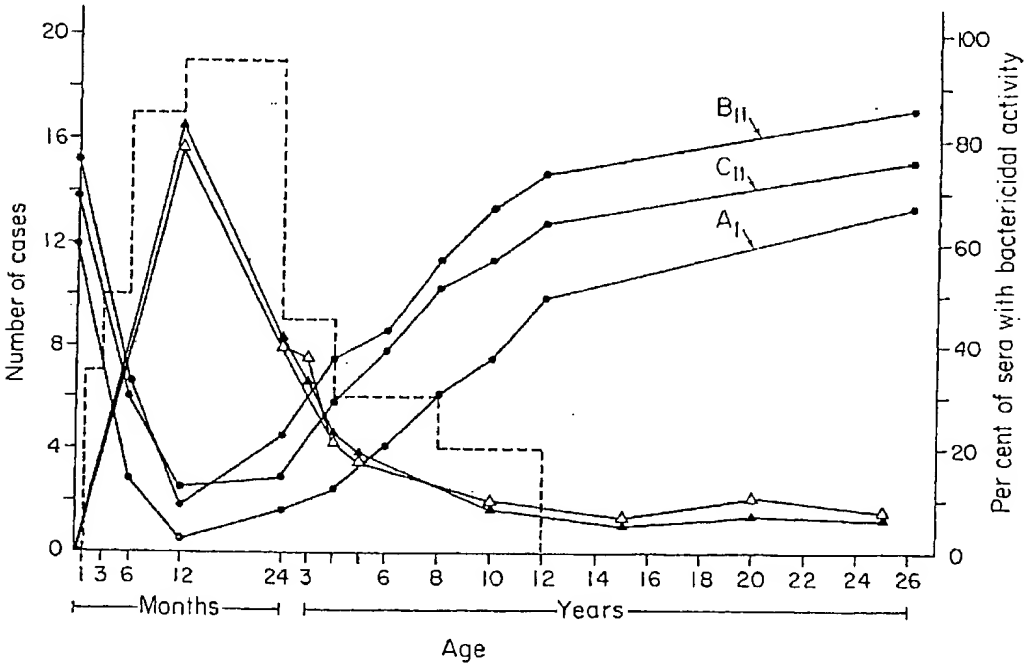

FIG. 1. Age-related incidence of meningococcal disease in the United States and prevalence of serum bactericidal activity against three pathogenic strains of *N. meningitidis*. ▲——▲, number of cases/100,000 age-specific population, 1965; △——△, number of cases/100,000 age-specific population, 1966; ———, age distribution of 72 cases admitted to Los Angeles Children's Hospital 1944–1953 (adapted from Smith [39]); ●——●, per cent of sera in each age group having a bactericidal titer of 1:4 or greater against meningococcal strains A1, B11, and C11. Sera from 282 children (at least 20 in each age group) and 567 army recruits (ages 19 to 26 yr) were tested in the presence of exogenous complement. Each point in the figure represents the incidence of disease or prevalence of bactericidal activity among subjects in the age range encompassed by it and the previous point (e.g., 2.5% of children 6–12 months of age had serum bactericidal activity against meningococcal strain A1).

1953) (39) was included to provide more detailed data on the incidence of meningococcal disease during the first 2 yr of life. Inasmuch as two epidemics of meningococcal meningitis occurred between 1944 and 1953 (14), it is reasonable to assume that many of the cases among these children were caused by serogroup A (40). Cases occurring in 1965 and 1966 were due in the main to meningococcal serogroups B and C (40).

[2] Courtesy of Ida Sherman, Acting Chief, Statistics Section, Epidemiology Program.

The data in Fig. 1 show that meningococcal disease is uncommon in the 1st month of life, but that significant numbers of cases begin to appear during the 2nd and 3rd months. The peak incidence of meningococcal meningitis occurs between 6 months and 2 yr of age, after which there is progressive decrease in attack rate. A second, smaller peak occurs in the young adult population. This is due almost entirely to cases of meningococcal disease among military recruits (42–44). After age 25, the attack rate per year fluctuates between 0.3 and 0.5/100,000 population.

The per cent of individuals having bactericidal activity in their serum against the prototype strains of *N. meningitidis* is inversely proportional to the incidence of meningococcal meningitis during the first 12 yrs of life. Results in Fig. 1 show that sera from more than 50% of newborn infants have bactericidal titers of at least 1:4 against one or more of the meningococcal test strains. The prevalence of bactericidal activity decreases rapidly after birth and reaches its lowest level between 6 months and 24 months of age. From 24 months to 12 yrs of age, there is an essentially linear increase in per cent of sera having bactericidal activity. By ages 19–26 yr, serum from approximately 67% of incoming military recruits has bactericidal activity against the serogroup A meningococcus, 86% against serogroup B, and 76% against serogroup C. These figures are only slightly higher than those for serum from newborn infants.

Serum Bactericidal Activity and Susceptibility to Systemic Meningococcal Disease.—It seems reasonable to hypothesize, on the basis of data presented in Fig. 1, that susceptibility to meningococcal meningitis is related to a deficiency of humoral antibodies as measured by the serum bactericidal test (45, 46). In order to test this hypothesis, a prospective study was started among incoming recruits at the U.S. Army base, Fort Dix, N. J. Recruits were bled during the 1st wk of basic training and the sera stored at −70°C in anticipation of the occurrence of meningococcemia or meningococcal meningitis among some of the men during the 8 wk training period. This serum is referred to as *base line serum*, as opposed to acute phase serum collected on the day of onset of clinical disease (often after parenteral administration of antibiotics). In all, 14,744 recruits were bled between 1 December 1967 and 31 March 1968, among which 60 cases of systemic meningococcal disease occurred. In every instance in which the organism was isolated, it belonged to serogroup C. At the peak of the meningitis outbreak in February, the attack rate reached 0.5 per 100 recruits per 4 wk training—a rate approximately 300 times greater than that current in the civilian population of the United States (47).

Base line serum from each case was tested for bactericidal activity against the strain of meningococcus isolated from his blood or cerebrospinal fluid (homologous strain). 10 control base line sera, selected at random from men in the same training platoon as the case, were also tested against the meningococcal isolate. There were 46 instances in the prospective study in which both base line serum

and the infecting meningococcal strain were available for study. In addition, base line sera from five group C cases of meningitis which occurred at Fort Dix, winter 1966 and spring 1967, and sera from three cases occurring in April 1968 were included, along with appropriate control sera. Results of this study are summarized in Table II.

It is clear from the data that base line sera from cases of systemic meningococcal disease lack bactericidal activity to the homologous disease-producing strain of meningococcus. Only 5.6% of base line sera from cases had bactericidal

TABLE II

Meningococcocidal Activity of Base Line Sera from Prospective Cases of Meningococcal Disease*

Meningococci	Bactericidal titer 1:4 or greater		Statistical significance
	Base line sera from cases	Base line sera from controls	
	No. positive/total (%)		
Homologous strains‡	3/54 (5.6)	444/540 (82.2)	$P < 0.001$§
Other pathogenic strains			
A1 (serogroup A)	4/23 (17.4)	166/230 (72.2)	$P < 0.001$‖
B11 (serogroup B)	3/23 (13.0)	179/230 (77.8)	$P < 0.001$‖
C11 (serogroup C)	2/23 (8.7)	154/230 (67.0)	$P < 0.001$‖
Strain from carrier (G-2-81) (Serogroup Y)	21/23 (91.3)	221/230 (96.1)	$P > 0.750$‖

* Obtained from recruits in 1st wk of basic training at Fort Dix, N. J., 1967–1968.

‡ Isolated from patients whose base line sera were tested. Base line serum from each case of meningococcal disease was tested against his own meningococcal strain. Base line sera from 10 controls were tested against each strain (total 540 control sera).

§ Calculated using Fisher's exact test for 2 × 2 tables (63).

‖ Calculated by the method of χ^2 (63).

activity in dilutions of 1:4 compared to 82.2% of control sera. Further, results in Table II indicate that base line sera from cases are relatively deficient in their ability to kill disease-producing strains of meningococci in general. Thus, the proportion of base line sera from cases able to kill prototype strains A1, B11, and C11 was significantly lower than that of controls. The mean titer of control sera having bactericidal activity against meningococcal strain A1 was 1:16 (range 1:4 to 1:128). However, when tested against meningococcal strain G-2-81 (serogroup Y) isolated from the nasopharynx of an asymptomatic carrier, base line sera from cases killed as frequently as did control sera.

Immunological Deficit in Sera from Susceptible Host Population.—There are several possible explanations for the inability of base line sera from cases of meningococcal meningitis to kill their own and other disease-producing strains

of meningococci. The sera could be deficient in antibody to the respective strains of meningococci by lack of previous (or recent) contact with relevant antigens; or as a result of a more general defect in immunoglobulin production such as hypogammaglobulinemia or dysgammaglobulinemia. There could be inhibitors of the bactericidal reaction, abnormalities in complement components, or absence of ancillary factors necessary for the bactericidal reaction. The following experiments were performed to distinguish which of these defects is the one most commonly observed.

Base line sera from cases of meningococcal meningitis are deficient in antibodies to pathogenic strains of meningococci. This was shown by an experiment in which antimeningococcal antibodies (IgG) to prototype strain C11 (serogroup C) were measured by indirect immunofluorescence. Antibodies to strain C11 were not found among 20 of 23 base line sera from cases of group C meningitis. 21 of these sera lacked bactericidal activity to C11 (Table II). However, 23 bactericidal sera from controls each had detectable antimeningococcal antibodies to strain C11 (immunofluorescent titer 1:2 or greater).

Three cases in Table II had bactericidal activity in their base line sera to their own meningococcal isolates. The cause of this lethal activity is not known. No immunoglobulins from classes G, M, or A were found by immunofluorescence to be directed against the homologous meningococcal strains. In contrast, of 10 control sera having bactericidal activity to these meningococcal strains, all had antimeningococcal antibodies (10, IgG; 6, IgM; and 3, IgA).

Base line sera from 23 cases had an average of 169.5 $C'H_{50}$ units/ml (range 162.2 to 186.5 units) control sera 176.8 $C'H_{50}$ units/ml (range 157.9 to 189.4 units), both within the established normal range for the procedure (34).

Sera from susceptible individuals do not appear to lack factors other than antibody which are necessary for bactericidal activity against meningococci, nor do such sera contain inhibitors of the bactericidal reaction (e.g., blocking antibodies, anticomplementary substances). As seen in Table II, 21 of 23 base line sera from cases of meningitis were bactericidal to meningococcal strain G-2-81. Further, such sera were fully able to restore the ability of purified gamma globulin to kill pathogenic strains of meningococci. Table III records the results of an experiment in which a standard amount of base line serum was added in lieu of complement to serially diluted pooled human gamma globulin (Cohn fraction II; 10 mg/ml). In seven of eight instances, the bactericidal titers against the prototype C strain were essentially the same whether base line serum from cases or the standard complement source was used to supply the effector mechanism for bactericidal activity. In the one exception (No. 7123), the complement titer was below normal.

The data indicate that individuals who are susceptible to systemic meningococcal disease lack humoral antibody to the offending strain of meningococcus (as well as to other case strains). However, the results in Table IV show that

such individuals are capable of responding immunologically during the course of infection. Bactericidal titers against homologous strains of meningococci were determined in convalescent sera obtained from 11 cases of meningitis which occurred at Fort Dix, N. J., in 1968. The sera were drawn at least 1 wk after cessation of antibiotic therapy (usually potassium penicillin). In each instance, there was a marked increase in bactericidal activity, reciprocal titers ranging from 256 to 2048. Base line serum from these cases had titers of less than 1:4 (Table II).

TABLE III

Reconstitution of Bactericidal Activity of Human Gamma Globulin by Sera from Prospective Cases of Meningococcal Disease*

Sera		Reciprocal bactericidal titer‡	
Description	Code No.	Serum	Serum (1:4) + gamma globulin§
Base line sera from prospective cases of meningococcal disease	2,651	<4	256
	6,884	<4	128
	7,123	<4	32
	8,860	<4	256
	9,587	<4	128
	10,325	<4	128
	10,662	<4	128
	12,059	<4	128
Complement (P. M.)		<4	128

* Base line sera from recruits in 1st wk of training at Fort Dix, N. J., 1967–1968.

‡ Tested against meningococcal strain C11 (serogroup C).

§ Cohn fraction II (10% w/v) diluted serially in twofold steps. Serum added to final dilution of 1:4. Gamma globulin had no bactericidal activity by iteslf.

The rise in bactericidal activity in convalescent serum is reflected by the appearance of IgG, IgM, and IgA antibodies to the patient's own strain of meningococcus. Immunoglobulin titers were measured by indirect immunofluorescence in acute and convalescent sera from four cases of meningitis. As shown in Table V, there was a marked, specific rise in level of the three classes of immunoglobulin within 2 wk of onset of disease. It is of interest that there were no detectable antibodies to the homologous meningococcal strains in acute sera from these four cases.

Fate of Susceptible Host Population.—While it is evident that base line sera from cases of meningococcal disease lack bactericidal antibody to the causative organism, it is also true that serum from as many as 35% of young adult males is unable to kill disease-producing strains of meningococci. Why, then, is the attack rate of meningococcal disease so low (less than 1%), even under

epidemic conditions such as existed at Fort Dix in the winter of 1967–68? Three possibilities exist, none of which are mutually exclusive: (*a*) The majority of susceptibles are not exposed to meningococci of proved pathogenicity; (*b*) factors other than circulating antibody predominate in defense against systemic meningococcal disease; (*c*) subclinical cases of meningococcemia occur.

An intensive study of three basic training companies at Fort Dix, N. J., in 1968 provides information on these points. The object was to determine the number of men in each training group who acquired pathogenic strains of men-

TABLE IV

*Bactericidal Activity of Convalescent Sera from Cases of Meningococcal Disease**

Sera		Onset of disease	Reciprocal bactericidal titer‡
Code No.	Date obtained		
A-612	12 April	11 March	1024
A-616	4 April	9 March	512
A-635	23 April	25 March	512
A-637	20 April	22 March	1024
A-640	28 March	28 February	512
A-645	5 April	8 March	2048
A-651	9 April	12 March	1024
A-656	4 April	25 February	1024
A-668	12 April	12 March	1024
A-670	17 April	18 March	512
A-677	6 April	13 March	256

* Fort Dix, N. J., 1968. All cases caused by group C meningococci.

‡ Serum from each patient was tested against his own meningococcal isolate. Exogenous complement was added. Titers of base line sera to the homologous strains were less than 1:4 (Table II).

ingococci against which they lacked serum bactericidal activity. Summarized data from this study are presented in Table VI.

492 men from the three companies were bled and had nasopharyngeal cultures taken during the 1st, 3rd, 5th, and 7th wk of basic training. Five proved cases of meningococcal meningitis, all due to sulfonamide-resistant, serogroup C organisms, occurred during the study period; three in E-5-3 Company (March), one in E-5-3 Company (April), and one in E-2-3 Company (April). The two E-5-3 companies were completely independent training companies. One additional case of meningitis in E-2-3 Company (April) was suspected to be meningococcal in origin, but was not confirmed bacteriologically.

Bactericidal activity of the 1st wk serum from each recruit was tested against the meningococcal strain(s) isolated from the case(s) within his training company. Results in Table VI show that 54 of 492 (11%) recruits had bactericidal titers less than 1:4 against the case strains. This group is defined as the suscepti-

TABLE V

Antimeningococcal Antibodies of Immunoglobulin Classes G, M, and A in Sera from Recruits with Meningococcal Disease‡*

Sera			Reciprocal immunofluorescence titer§		
Patient	Code No.	Date obtained	IgG	IgM	IgA
J. H.	A-199	11/19/66‖	<2	<2	<2
	A-200	11/25/66	128	64	32
C. C.	A-321	1/16/67‖	<2	<2	<2
	A-322	1/23/67	128	128	64
C. S.	A-420	3/16/67‖	<2	<2	<2
	A-421	3/24/67	64	64	32
S. C.	A-428	4/21/67‖	<2	<2	<2
	A-430	4/28/67	512	512	128

* Determined by indirect immunofluorescence.
‡ Fort Dix, N. J. All cases caused by group C meningococci.
§ Sera tested against the patient's own meningococcal isolate.
‖ Date of onset of disease.

TABLE VI

Incidence of Meningococcal Disease among Susceptible Recruits at Fort Dix, N. J., 1968

Training Company	Sera lacking bactericidal activity to case strains*	Susceptible population‡			
		Meningococcal isolates§		Bactericidal activity to acquired C_1 strain	Incidence of meningococcal disease
		No. positive/total	No. serogroup C		
	No. negative/total			*No. positive/total*	*No. cases/No. exposed susceptibles (%)*
E-5-3 (March)	30/195	28/30	16	8/16	3/8 (37.5)
E-5-3 (April)	13/185	8/13	3	1/3	1/2 (50.0)
E-2-3 (April)	11/112	8/11	5	2/5	1/3‖(33.3)
Total.....	54/492	44/54	24	11/24	5/13 (38.5)

* All cases caused by sulfa-resistant, group C meningococci.
‡ Individuals lacking serum bactericidal activity to disease-producing strains of meningococci prevalent in their basic training companies.
§ From carriers and cases.
‖ An additional case of meningitis occurred, but the etiological agent was not identified.

ble host population, i.e., the population at highest risk to systemic disease from meningococcal strains of proven pathogenicity within the immediate environment.

44 of the 54 presumed susceptible individuals became carriers of a meningo-

coccus after the 1st wk of training. Of these, 24 (44.4% of the original 54) acquired a sulfonamide-resistant, group C meningococcus. However, serum from 11 of the men had bactericidal activity against the acquired group C strain, indicating that their organisms were different from the case strains. Thus, of the original 54 susceptibles, only 13 were exposed to meningococci compatible with the prevalent pathogenic strains and to which they had no bactericidal activity. The five (and possibly six) cases of meningitis occurred among this group—an incidence of 38.5%.

DISCUSSION

Susceptibility to meningococcal disease in man is related to a selective deficiency of antibody to the offending organism. This conclusion derives from two experiments in which susceptibility to infection and bactericidal activity of serum were compared. In the first experiment, it was found that the age-specific incidence of meningococcal disease in the general population of the United States is inversely proportional to the prevalence of antimeningococcal bactericidal antibodies in the serum. In the second experiment, a study was initiated among military recruits to measure the bactericidal activity of sera from prospective cases of meningococcal disease. The results show that base line sera from cases lack antibodies to the homologous meningococcal strains. Such serum is not only deficient in antibodies to the patient's own strain, but to heterologous disease-producing strains as well—suggesting a lack of immunity to pathogenic meningococci in general. The lack of antimeningococcal antibodies in base line sera from cases was confirmed by indirect immunofluorescence.

3 of 54 sera from prospective cases of meningitis *did* killl homologous and heterologous strains of meningococci. However, neither gamma G, M, nor A antimeningococcal antibodies were present as judged by immunofluorescence. The cause of the bactericidal activity in these three sera is thus unknown.

The deficiency in sera from susceptible hosts is confined to antibodies to meningococci. Hemolytic complement levels are normal, and addition of exogenous complement does not increase bactericidal activity.[1] The sera are fully able to effect bactericidal reactions against meningococcal case strains when reconstituted with purified human gamma globulin (Cohn fraction II). Furthermore, unsupplemented base line sera could kill a group Y strain of meningococcus which was isolated from an asymptomatic carrier.

While sera from susceptible hosts lack antibody to the offending meningococcus, such individuals are capable of initiating an immune response to meningococcal antigens. Thus, convalescent sera from cases of meningitis have markedly increased contents of IgG, IgM, and IgA antibodies to their own meningococcal strain. This increase in antibody titer is accompanied by a sharp rise in bactericidal activity. That the deficiency of antimeningococcal immunoglobulins in sera of susceptibles is not part of a generalized immunological deficiency syndrome is shown by normal total immunoglobulin concentrations in acute

sera from cases (48) and by the absence of a clinical history of unusual susceptibility to other infectious diseases.

Two previous studies have suggested that the prevalence of antimeningococcal antibodies in blood from normal individuals increases as a function of age of the donor. Matsunami and Kolmer (19) titrated the bactericidal activity of whole blood from 26 children, ages 6 months to 10 yr, and from normal adults. They concluded that the blood from children was "somewhat less" meningococcocidal than that from adults. Silverthorne and Fraser (49) also compared bactericidal activity of blood from children and adults. 23 of 33 blood samples from adults were positive against a pathogenic strain of meningococcus, compared to 0 of 11 samples from children less than 2 yr old. Both of these studies suggested that children several months to 2 yr of age lack serum bactericidal antibodies to pathogenic meningococci, whereas most adults have such antibodies. This is consonant with the findings of the present study. In addition, these earlier experiments suggested that young children also lack opsonizing antibodies to meningococci.

There is considerable precedent in the literature of infectious diseases for relating susceptibility to systemic disease to absence of humoral antibodies. Many of the so-called "diseases of childhood" have a reciprocal relationship between the curves formed by age-related incidence of infection and presence of antibacterial or antitoxic antibodies in serum (13). Perhaps the most striking example is that of meningitis caused by *Haemophilus influenzae*. Fothergill and Wright (50) found an inverse relationship between incidence of *H. influenzae* meningitis and bactericidal power of blood almost identical with that shown for *N. meningitidis* in the present study. Not only did the percentage of individuals with sera capable of killing the organism increase progressively after age 3 yr, but the mean bactericidal potency of their sera also increased. Other diseases of childhood in which the development of immunity has been shown to be directly related to a progressive increase in prevalence of specific humoral antibodies with age are diphtheria (51), scarlet fever (52), poliomyelitis (53), and mumps (54).

The strongest evidence that antimeningococcal antibodies in "normal" serum are protective against systemic meningococcal disease is the observation that meningococcal meningitis is unusual in the neonatal period (39, 55, 56) but increases with the onset of physiological hypogammaglobulinemia (57). Analogy with the diseases of childhood cited above suggests that this is due to the transient protective effect of passively transferred maternal antibody (58). The successful use of hyperimmune animal serum in the treatment of systemic meningococcal disease (59) supports the thesis that antimeningococcal antibodies in serum of normal human beings are protective. Bactericidal and opsonic antibodies (60) were present in these therapeutic sera; such antibodies have been described in normal human sera (19–22).

Work in nonimmunized animals also suggests a protective role for "natural" humoral antibodies against meningococci. Matsunami and Kolmer (19), using a single strain of meningococcus, showed that the relative abilities of several types of laboratory animals to survive challenges with graded doses of bacteria correlated with the bactericidal titers of whole blood against the organisms. Recently, Evans et al.[3]

[3] Evans, J. R. Personal Communication.

demonstrated a positive correlation between the abilities of numerous group B strains of meningococci to survive in normal rat serum in vitro and their abilities to cause disease in the rat in vivo.

It is important to emphasize that results of the present experiments are not interpreted to indicate that serum bactericidal activity per se is the protective factor in natural immunity against meningococcal disease. The serum bactericidal test was used only as a sensitive indicator of specific antibodies to meningococci. Such antibodies may have other functions in addition to bactericidal activity (e.g., opsonization); or other, nonbactericidal, antibodies may play a role. Indeed, there is no evidence that humoral antibodies are the sole, or even the major host defense mechanism. Phagocytic cells of the reticuloendothelial system, for example, are undoubtedly of importance in confining and eliminating the meningococcus, and local factors in the nasopharynx may also contribute. Nevertheless, it does seem clear from the present experiments that a deficiency of circulating antimeningococcal antibodies is firmly associated with the establishment of meningococcemia. Epidemiological observations support this conclusion. Thus, while people with humoral antibodies against meningococci frequently become carriers of the organisms,[1] they rarely become cases (6). On the other hand, people who become cases rarely harbor the meningococcus in the nasopharynx more than a few days prior to onset of systemic disease (6, 61). This is presumably because, in susceptible individuals, invasion of the deeper tissues and blood occurs before the carrier state can be established. The relation between the factors that control the carrier state and humoral protective antibodies is not known. However, a later report in this series (62) shows that systemic immunization with meningococcal products can affect local defenses to the meningococcus.

It has been established that meningococcal meningitis is a disease which is spread by asymptomatic carriers (1, 2). Numerous studies have suggested that the incidence of clinical disease is related to the introduction and spread of pathogenic strains of meningococci in the population (1, 7). Our studies suggested that, even during an epidemic, meningococcal disease occurs in only a fraction of the predicted susceptibles. An attempt was made, therefore, to determine the fate of the individuals comprising the susceptible population during an epidemic of group C meningitis at Fort Dix, N.J.

In a group of 492 basic recruits in three training companies, 54 were found to lack serum bactericidal activity against the prevalent disease-producing strains. 44 of this presumed susceptible group acquired a meningococcus during the 8 wk of training, but in only 13 instances were the organisms compatible with the case strains. Of the 13 exposed susceptibles, 5 developed systemic meningococcal disease—an incidence of 38.5% of susceptibles, but only 1% of the total population. These findings indicate that the majority of susceptible individuals

escaped clinical infection because they were not exposed to the prevalent strains of pathogenic meningococci.

It is not surprising that all the potentially susceptible individuals did not become cases upon exposure to the pathogenic strains of meningococci. The fact that base line sera from cases of meningitis have bactericidal titers of less than 1:4 does not mean that all people with titers below this level are susceptible to infection, although many obviously are. Furthermore, other factors, such as intensity of exposure to pathogenic organisms, may be more instrumental in determining the incidence of diseases than exposure per se. Finally, it is possible that in some instances subclinical meningococcemia occurred and was not detected.

SUMMARY

Susceptibility to systemic meningococcal disease is related to a selective deficiency of humoral antibodies to pathogenic strains of meningococci. In a study of the age-specific incidence of meningococcal meningitis in the United States, it was found that the proportion of individuals with serum bactericidal activity to meningococci of serogroups A, B, and C was reciprocally related to the incidence of disease. The prevalence of bactericidal activity was highest at birth and among adults, and lowest in infants between 6 and 24 months of age.

Sera from 51 of 54 prospective cases of meningococcal disease among military recruits were deficient in antibodies to homologous and heterologous strains of pathogenic meningococci as determined by serum bactericidal activity and indirect immunofluorescence. Such sera, however, could support the bactericidal activity of purified human gamma globulin (Cohn fraction II), and such individuals could respond immunologically to infection with meningococci. The implication is that susceptible persons are deficient in antimeningococcal antibodies because they have not received significant exposure to meningococcal antigens in the past.

The fate of individuals who lack bactericidal antibodies to pathogenic meningococci was determined during an outbreak of group C meningitis among military recruits. The incidence of disease was found to be primarily associated with the incidence of exposure of susceptibles to the pathogenic strains. Whereas 81.5% of the presumed susceptibles acquired a meningococcal strain, only 24.1% acquired an organism similar to the prevalent disease-producing strains. Of the exposed susceptibles, 38.5% developed systemic meningococcal disease.

We wish to thank those physicians listed in Table I for their help in obtaining sera from children. SP5 K. O'Brien and SP5 J. Funk assisted in collecting the sera from recruits. Colonel T. H. Lamson and Major W. Morgan helped coordinate the collection of sera and bacteriological specimens at Fort Dix, N. J. Mr. C. Harkins supervised the diagnostic bacteriology. We are indebted to SP5 J. Wilczek for invaluable technical assistance in performance of the bactericidal and immunofluorescence tests.

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Exhibit 2

Serological Basis for Use of Meningococcal Serogroup C Conjugate Vaccines in the United Kingdom: Reevaluation of Correlates of Protection

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The antibody data supporting the use of meningococcal serogroup C conjugate (MCC) vaccines in the United Kingdom were generated by serum bactericidal assay (SBA) using rabbit complement (rSBA). This may give higher titers than those obtained with human complement (hSBA), for which the "gold standard" correlate of protection for meningococcal C disease is a titer of ≥4. Comparison of rSBA and hSBA titers in sera from unvaccinated adults with an rSBA titer of ≥8 showed that for 93% (27 of 29) the titer was ≥4 by hSBA, confirming natural protection. Furthermore, sera from MCC vaccinees showed that an rSBA titer of <8 or ≥128 discriminated susceptibility and protection well (85% with rSBA titers of <8 had hSBA titers of <4, and 99% with rSBA titers of ≥128 had hSBA titers of ≥4). However, discrimination was poor in the rSBA titer range 8 to 64, with only 60% having hSBA titers of ≥4. In such cases we propose that protection can be assumed if there is a fourfold rise in titer between pre- and postvaccination sera or if there is a characteristic booster response to a polysaccharide challenge dose with, if available, evidence of antibody avidity maturation or an hSBA titer of result ≥4. Applying these criteria to toddlers, 10 to 40% of whom had titers in the range 8 to 64 after a single dose of MCC vaccine, showed that 94% had a fourfold rise in titer, including 98% of those in the titer range 8 to 64. In addition, of those with titers of <128 post-MCC vaccination, 90% had titers of ≥128 after a 10-μg polysaccharide booster dose, compared with only 7% of unprimed age-matched toddlers given a full 50-μg dose. Furthermore, the increase in geometric mean avidity index pre- and postbooster was independent of post-primary MCC titer. These results indicated that the majority of toddlers with an rSBA titer between 8 and 64, and some of those with an hSBA result of <4, have mounted a protective immune response with the induction of immunological memory.

Meningococcal serogroup C (MenC) conjugate (MCC) vaccines have been extensively evaluated in phase I and II trials in the United Kingdom (7, 15, 31–34) and elsewhere (2, 10, 11, 23, 24, 36) and have been shown to be highly immunogenic, generating functional antibodies as measured by serum bactericidal assay (SBA). Studies of military recruits during the 1960s had shown that those with naturally acquired SBA titers of ≥4 were protected from MenC disease (20). The United Kingdom Medicines Control Agency therefore took the view that efficacy trials would not be required for MCC vaccines but instead serological correlates utilizing SBA could be relied upon. A similar rationale has also been used to license unconjugated MenC (and MenA) polysaccharide vaccines. Antibody responses to these capsular polysaccharide vaccines have been measured by various serological methods though licensure was gained using data obtained by radioimmunoassay and SBA in the United Kingdom. The original licensure in the United States in the 1970s, however, was based upon classic randomized efficacy trials (4, 16). Serogroup C polysaccharide vaccines have been shown to be efficacious in those over 2 years of age and to reduce carriage of serogroup C meningococci in the short term (4, 12), although they are poorly immunogenic and not protective in those under 2 years of age (35).

The original serological correlate of protection in military recruits was obtained using an SBA in which human sera was the exogenous complement source (hSBA). However, large volumes of suitable human source complement preserved sera are not available or even practical to obtain due to the occurrence of naturally occurring antimeningococcal antibodies induced by oropharyngeal carriage of meningococci or other neisserial species. In the absence of a commercial source of human complement, the standardization of assays between laboratories, each of whom is using their own in-house source, is difficult.

Commercially available heterologous complement has the advantage of being manufactured and supplied to a high standard of consistency and offers the only practical way of achieving standardization of SBA results between laboratories. Therefore, 3- to 4-week-old baby rabbit serum is now recommended as an alternative complement source for the SBA (26, 37).

It is generally accepted, however, that serogroup C meningococci are more susceptible to serogroup C-specific antibodies when using baby rabbit complement as opposed to human complement, resulting in higher SBA titers (21). With the reliance on serological correlates of protection for licensure of MCC vaccines, we have evaluated the use of SBA titers gen-

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TABLE 1. Comparison of the numbers of sera positive and negative by human complement stratified by rabbit complement titer for subjects who have received MCC vaccination and for unvaccinated adults

rSBA titer	No. of sera with indicated hSBA titer in:			
	MCC vaccinees		Unvaccinated adults[a]	
	<4	≥4	<4	≥4
<8	34	6		
8	3	0	0	2
16	3	3	0	2
32	2	5	2	4
64	4	10	0	5
128	1	26	0	4
≥256	0	97	0	10
Total	47	147	2	27

[a] Note that only those adults with an rSBA titer of ≥8 were tested by the hSBA.

erated with rabbit complement (rSBA) with the aim of defining rSBA responses that predict protection against MenC disease. The objective is to address concerns about specificity without introducing unnecessarily stringent criteria that might obstruct the licensure and use of MCC vaccines elsewhere.

MATERIALS AND METHODS

MCC vaccine. The MCC vaccines used in this study were the Meningitec vaccine, which contains 10 μg of MenC oligosaccharide coupled to CRM_{197} mutant diphtheria toxin (Wyeth Lederle Vaccine & Pediatrics [WLVP], Pearl River, N.Y.), the Menjugate vaccine, which contains 10 μg of MenC oligosaccharide linked to CRM_{197} mutant diphtheria toxin vaccine (Chiron Vaccines, Siena, Italy) and the Neis-vac, a de-O-acetylated MenC polysaccharide (10 μg) coupled to tetanus toxoid (Baxter Hyland Immuno, formerly North American Vaccines Inc. [NAVA], Beltsville, Md.). Booster doses at 6 months following MCC vaccination comprised a 0.1 ml dose of licensed meningococcal AC polysaccharide (MACP) vaccine [Mengivac (A+C); Pasteur Merieux, Lyon, France] containing 10 μg each of MenA and MenC polysaccharides.

Study groups. The study groups were as previously described, comprising infants (34), toddlers (12 to 14 months) (31), preschool children (3 to 4 years) and school leavers (14 to 17 years) (E. Miller, P. Richmond, R. Borrow, E. Kaczmarski, K. Cartwright, R. Morris, and C. Thornton, Abstr. 11th Int. Pathog. Neisseria Conf., p. 57, 1998), 18 to 26 year olds (33), and laboratory staff (R. Borrow, E. Miller, N. Peake, R. Rahim, N. Andrews, M. Acuna, S. Martin, J. Southern, and E. B. Kaczmarski, submitted for publication) who were participants in trials of MCC vaccines in the United Kingdom. The postvaccination serological data in this paper are restricted to recipients of the WLVP MCC vaccine with the exception of the toddlers, for whom data for all three manufacturers' vaccines are included. All study sera were assayed by rSBA as part of the respective trials, with selective sera being reassayed by hSBA.

SBA. The SBA for infant, toddler, and 18- to 26-year-old cohorts was performed as described previously (2) using broth culture for growth of target strains prior to the SBA assay. The SBA for preschool and school leaver cohorts was performed as described by Maslanka et al. (26), in which agar culture was utilized for target strain growth. The complement source used was either 3- to 4-week-old baby rabbit complement (Pelfreeze Biologicals, Brown Deer, Wis.) or complement-preserved human serum collected from a subject with no intrinsic SBA activity against strain C11. The target strain used throughout was C11 (C:16:P1.7^a,1). SBA titers were expressed as the reciprocal of the final serum dilution causing ≥50% killing at 60 min. For computational purposes and the calculation of fourfold rises. SBA titers of <4 were given a value of 2.

Avidity indices. Serogroup C-specific immunoglobulin G avidity was tested by an elution enzyme-linked immunosorbent assay, using the chaotrophe thiocyanate as described elsewhere (18) and modified for the MenC assay (32).

Statistical methods. rSBA and hSBA titers determined for the same sera were compared by calculating the proportion with protection by hSBA at different rSBA titers with 95% confidence intervals (95% CIs). Specificity within a population of unvaccinated infants and toddlers was also calculated with a 95% CI. 95% CIs were also calculated for the geometric mean avidity index.

RESULTS

Comparison of rSBA and hSBA titers for the same sera. Paired SBA titers generated with both rabbit and human complement were generated on a total of 194 post-MCC vaccination serum samples (Table 1). These comprised sera from infants after single or multiple doses of MCC (34) as well as those from toddlers after a single dose (31). An rSBA titer of <8 or ≥128 discriminated well between susceptibility and protection as defined by the "gold standard" hSBA cutoff, since 34 of 40 (85% [95% CI, 70 to 94]) of sera with an rSBA titer of <8 had an hSBA titer of <4 and 123 of 124 (99% [95% CI, 96 to 99.9%]) with an rSBA titer of ≥128 had an hSBA titer of ≥4. However, discrimination was less good in the rSBA titer range 8 to 64, with only 18 of 30 (60% [95% CI, 41 to 77%]) showing a titer of ≥4 by hSBA. Although all three sera with titers of 8 by rSBA had titers of <4 by hSBA, this titer was included in the equivocal region because only three paired results were available. Sensitivity and specificity are not calculated for the post-MCC vaccination sera because they are highly dependent on the proportion of samples that are in the rSBA titer range 8 to 64, which varies depending on the age and dose (Table 2).

In unvaccinated adults with rSBA titers of ≥8 (Table 1), 27 of 29 (93%) had an hSBA titer of ≥4. Of these 27, only 14 (52%) had an rSBA titer of ≥128. This shows that a high proportion of individuals with natural exposure have an rSBA titer in the equivocal range of 8 to 64. It also shows that in a population of unvaccinated adults, an rSBA of 8 to 64 predicts positivity (natural exposure) well with 13 of 15 also positive by hSBA (87% [95% CI, 60 to 98%]).

Age groups in which equivocal rSBA results occur after MCC vaccine. The rSBA responses in different age groups and with different immunization schedules using the WLVP MCC vaccine are shown in Table 2. Over 90% of infants have rSBA titers of ≥128 after two doses, with a similar proportion being found in 14 to 17 year olds and 18 to 26 year olds after a single

TABLE 2. rSBA titers 4 weeks after vaccination with MCC vaccine (10-μg dose)[a]

Vaccine manufacturer and age at first dose	No. of doses	No. tested	GMT[b]	No. (%) of subjects with indicated postvaccination rSBA titer:		
				≤4	8–64	≥128
Wyeth						
2 mo	1	50	33	16 (32)	15 (30)	19 (38)
2 mo	2 (1 mo apart)	56	776	1 (2)	3 (5)	51 (93)
2 mo	3 (1 mo apart)	53	1,011	1 (2)	1 (2)	51 (96)
12–14 mo	1	71	141	6 (8)	17 (24)	48 (68)
3–4 yr	1	87	908	1 (1)	10 (11)	76 (88)
14–17 yr	1	81	3,890	0 (0)	0 (0)	81 (100)
18–26 yr	1	86	1,425	0 (0)	6 (7)	80 (93)
Chiron						
12–14 mo	1	72	123	6 (8)	29 (40)	37 (52)
NAVA						
12–14 mo	1	72	564	0 (0)	7 (10)	65 (90)

[a] Data from United Kingdom trials.
[b] GMT, geometric mean titer.

dose. However, a substantial proportion of children under 5 years of age have rSBA titers in the equivocal titer range 8 to 64 after a single dose. The proportions of toddlers given a single dose of Chiron or NAVA MCC vaccine who had rSBA titers in the equivocal range were 40 and 10%, respectively (Table 2).

Resolution of the immune status of children with equivocal titers was essential in order to decide whether a one- or two-dose schedule was required for children of 1 to 4 years in the United Kingdom catch-up MCC immunization program.

Specificity of rabbit complement in the SBA in unvaccinated infants and toddlers. Prevaccination sera from 212 toddlers (31) and 54 infants (34) participating in MCC vaccine trials in the United Kingdom were tested by rSBA. None of these children had a history of prior meningococcal infection and should therefore be susceptible to MenC unless protective SBA antibodies were acquired through carriage or, in infants, through transfer of maternal antibodies.

Of the 212 toddler sera, 200 (94%) had rSBA titers of <4, with the remaining 12 distributed as follows: for six sera, titer = 4; for two sera, titer = 16; for three sera, titer = 32; and for one serum, titer = 512. For the 54 prevaccination infant sera, 46 (85%) had rSBA titers of <4; of the remaining 8 sera, six had titers of 4, one had a titer of 8, and one had a titer of 64. Thus, only one prevaccination toddler serum with a titer of 512 would have been categorized as protected based solely on the rSBA result being ≥128. The hSBA titer with human complement for this serum was 64, confirming protection. The specificity of the rSBA test in a population of unvaccinated uninfected individuals is therefore estimated to be 97% (258 of 265 [95% CI, 95 to 99%]) with a cutoff of ≥8 and 100% (265 of 265 [95% CI, 98.6 to 100%]) with a cutoff of ≥128. This shows that the rSBA does not measure nonspecific functional antibody in nonimmune subjects.

Fourfold rises. In the United Kingdom toddler study, 1 month after the primary dose of MCC vaccine, 94% of subjects, had a rise of four fold or more in rSBA titer compared with their prevaccination titer (for the Chiron vaccine, 91% [60 of 66]; for the WLVP vaccine, 89% [56 of 63]; for the NAVA vaccine, 100% [71 of 71]). For individuals in whom the post-MCC vaccine rSBA titer was between 8 and 64, 98% (50 of 51) had a fourfold rise in rSBA titer compared with prevaccination levels, regardless of the MCC vaccine administered.

Immunological memory responses to plain polysaccharide. In the United Kingdom MCC toddler study (31), the effect of a booster dose with 10 μg of C polysaccharide in toddlers previously immunized with MCC vaccine was investigated. For recipients of the Chiron, WLVP, and NAVA MCC vaccines, 47, 33, and 10%, respectively, had rSBA titers of <128 1 month after the first dose, but only 5, 6, and 3%, respectively, had titers of <128 postbooster (Table 3). For those with a postprimary SBA titer of <128, 90% had a postbooster titer of ≥128. This compares with 1 of 12 (8%) age-matched children (17 to 28 months) vaccinated with 50 μg of MACP vaccine as part of an outbreak control measure (8) ($P < 0.0001$).

Avidity indices for assessing immunological memory. Although the United Kingdom toddler study demonstrated a fall in rSBA titer, the avidity index was shown to increase from postprimary to preboost titers (Fig. 1). There was no significant difference in the avidity index when comparing subjects with postprimary SBA titers of <8, 8 to 64, or ≥128, the geometric mean avidity indices (95% CI) being 119 (90 to 158), 128 (118 to 138), and 128 (123 to 134), respectively, for all three vaccines combined.

TABLE 3. Proportion of toddlers with postbooster rSBA titers of ≥128 grouped by postprimary rSBA titer

Vaccine manufacturer	Proportion of toddlers with the indicated postprimary rSBA titer[a]:		
	<8	8–64	≥128
Chiron	2/3	22/24	34/34
WLVP	6/6	11/12	41/44
NAVA		6/7	62/63
Total (%)	8/9 (89)	39/43 (91)	137/141 (97)

[a] Results are given as number of toddlers with postbooster rSBA titer of ≥128/total number of toddlers.

Fourfold rises, response to boosting and avidity maturation according to post-MCC rSBA titers. In Table 4, the United Kingdom MCC toddler study participants were categorized by three different criteria: (i) by the presence of a fourfold rise in SBA titer pre-MCC to 1 month post-MCC, (ii) by the achievement of an rSBA titer of ≥128 after a 10-μg MACP booster, and (iii) by evidence of avidity maturation (i.e., rise in avidity index from 1 month post-MCC to pre-10-μg MACP booster 6 months later). There were no differences in the proportions meeting the above criteria between those with post-primary MCC titers of 8 to 64 compared and those with post-primary MCC titers of ≥128.

DISCUSSION

We have demonstrated the high specificity of the rSBA in young children who have not been previously vaccinated with MenC polysaccharide or MCC vaccines and could therefore be presumed to be susceptible to MenC infection. Therefore, rSBA titers of <8 predict susceptibility. This conclusion was corroborated in a recent university outbreak of MenC disease in which rSBA titers in students prior to or at the onset of invasive disease were all <4, suggesting that it is the absence of bactericidal activity is that is important irrespective of the complement source used (22). We have also shown that rSBA titers of ≥128 are highly predictive of protection but that many sera with rSBA titers in the range 8 to 64 also come from individuals with protection, particularly for adults with natural exposure. The main issue is therefore the interpretation of rSBA titers in the region of 8 to 64. For groups in whom there is likely to be a large proportion of results in this range, it is important to have other information in order to determine protection. We propose that the other information could be either an hSBA titer (the gold standard), a pre- and postvaccination sample pair that would enable the assessment of fold rises, or a post-polysaccharide booster result that can be compared with the primary response in age-matched controls.

We have shown that evidence of protection, as defined by a fourfold rise in rSBA titer with a characteristic booster response, may be present despite an hSBA titer of <4. We conclude, therefore, that while an hSBA titer of ≥4 indicates protection, an hSBA titer of <4 does not always indicate susceptibility. A similar conclusion was reached by Goldschneider

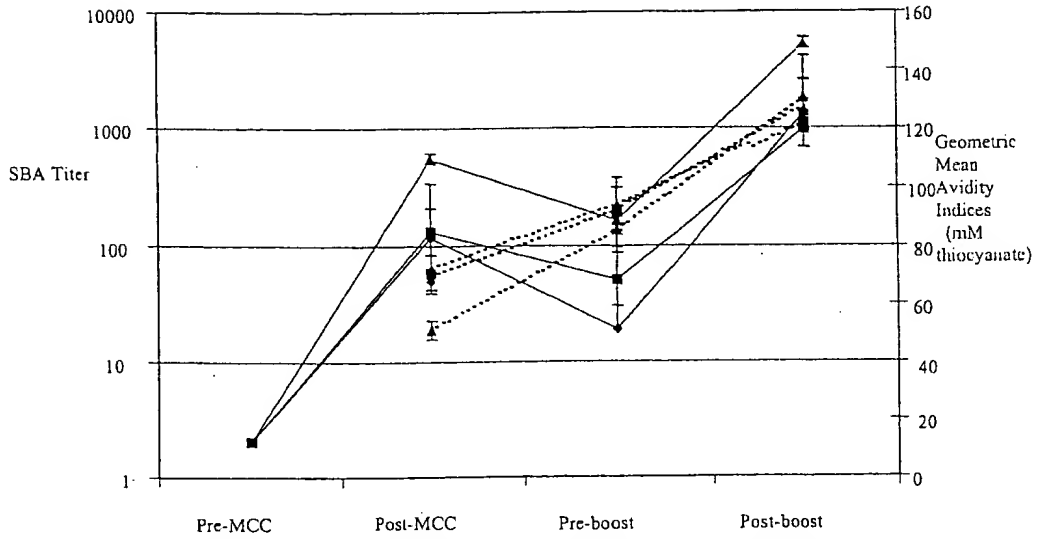

FIG. 1. SBA titers (solid lines) and antibody avidity (dotted lines) in toddlers receiving one dose of MCC followed by a 10-μg dose of MACP 6 months later. The three vaccines used in the study were manufactured by Chiron (diamonds), WLVP (squares), and NAVA (triangles).

et al. (20), who stated, "The fact that base line sera from cases of meningitis have bactericidal titers of less than 1:4 does not mean that all people with titers below this level are susceptible to infection, although many obviously are."

Fourfold rises in titer from pre- to postvaccination are widely used as measure of a significant response to vaccination, including the meningococcal polysaccharide vaccine (37). The World Health Organization specified the requirements for the meningococcal polysaccharide vaccine, advocating the use of 3- to 4-week-old baby rabbit serum as an exogenous complement source, and stated that "The antibody titers of the sera from at least 90% of the subjects should show a fourfold or greater rise after immunization." It is therefore already accepted that fourfold rises in serum bactericidal titers are indicative of the development of a protective response to unconjugated meningococcal vaccines, and extension of this notion to recipients of MCC would seem appropriate. Using this criterion, we showed that 98% of individuals with post-MCC titers of 8 to 64 should be protected against MenC disease.

Another indicator of long-term protection that is proposed is the SBA response to a polysaccharide booster. For toddlers with a postprimary SBA titer of <128, 90% had a postbooster titer of ≥128, compared with only 8% of age-matched naïve controls given a full 50-μg dose of C polysaccharide (8). This difference in response confirms the induction of immunological memory in the MCC vaccines despite postprimary SBA antibody levels in the equivocal range. Response to a plain polysaccharide boost has also been used to demonstrate immunological memory following *Haemophilus influenzae* type b (Hib) conjugate vaccination, even in the absence of a detectable primary response to the Hib vaccine (14, 19), and it is memory which is thought to contribute to the persistence of protection against Hib in United Kingdom children vaccinated only during infancy (6, 17).

There is now a growing consensus that evidence of successful induction of immunological memory by conjugate vaccines can be obtained by documenting avidity maturation in the months after primary vaccination with a further increase following a booster dose of plain polysaccharide antigen (1, 14, 18). By examining avidity maturation following a 10-μg booster dose of meningococcal polysaccharide, we have demonstrated that vaccinated individuals with SBA titers above and below 128 display similar avidity maturation in their antibody response and by this criterion should therefore have immunological memory established. Data now exist which demonstrate the increase in avidity over time following priming of infants or toddlers with Hib (18, 30), *Streptococcus pneumoniae* (3), and *Neisseria meningitidis* serogroup C conjugate vaccines (31). Such data have been generated in the context of boosters of low doses of plain polysaccharide or conjugate vaccine in young infants and are now accepted as indicative that a T-cell-dependent response has been generated.

Concerns have been raised about the relative protection afforded by immunological memory to the different organisms for which conjugate vaccines are available. SBA titers fall dramatically in young children after the primary series, with 25 of 53 (47%) infants with a three-dose schedule (34) and 16 of 65 (25%) toddlers with a one-dose schedule (31) of the WLVP vaccine having SBA titers of <8 6 to 10 months later. This suggests that, as with Hib conjugate vaccines (14), reliance on immunological memory rather than maintenance of SBA titers

TABLE 4. Categorization of United Kingdom MCC toddler sera post-primary MCC rSBA titers into numbers positive by fourfold rises, response to boosting, and avidity maturation

Post-MCC rSBA titer	Vaccine	No. with fourfold rise in titer from pre- to post-MCC/ total no. (%)	No. with rSBA titer of ≥128 post-10-μg MACP booster/ total no. (%)	No. with avidity maturation[a]/ total no. (%)
<8	All	0/11 (0)	8/9 (89)	3/5 (60)
8–64	All	48/49 (98)	39/43 (91)	25/27 (93)
≥128	All	116/117 (99)	137/141 (97)	78/86 (91)

[a] Rise in avidity from post-MCC to pre-MACP booster.

above a protective threshold will be necessary if MCC vaccines are to protect over the long term. In general, the pathogenesis of the infections caused by *N. meningitidis* involves invasion following nasopharyngeal colonization (9). Observations during epidemics (13, 20) and in infected laboratory workers (9) indicate that this period is usually short, though a recent case report demonstrated a 7-week interval (28). This suggests that local immunity, induced by carriage, is able to prevent invasion, but in naïve subjects, recent acquisition is associated with invasion because of the delay associated with the mounting of an effective primary response against the relevant strain. The length of this delay is unknown, but is likely to be different in naïve from that in subjects primed by MCC vaccine. For example, with Hib conjugate vaccines, a detectable antibody response post-polysaccharide boosting is present after 4 to 5 days (29), while responses following a primary series take up to 7 days (25). Pneumococcal conjugate vaccines (27), and even MenC polysaccharide vaccines (4, 12), reduce nasopharyngeal carriage of strains contained in the vaccine and therefore clearly provide a degree of mucosal immunity; this is further reinforced by recent pneumococcal conjugate vaccine efficacy data from the United States (5).

Until more data are available it is difficult to correlate the seriousness or speed of an invasive infection with the relative protection afforded by memory. Furthermore, it is likely that some degree of protection at the early stage of infection is mediated by factors other than antibody (such as complement). In addition, data on the seriousness of invasive infection have been correlated with host factors such as cytokine promoter polymorphisms, which may ultimately be more relevant to the outcome of infection. In the context of our current understanding of memory and protection, the theoretical concerns raised above do not justify unnecessarily stringent criteria for licensing and use of the current MCC vaccines. The United Kingdom postlicensure surveillance program will validate these immunological criteria against measures of clinical effectiveness over the coming years.

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Exhibit 3

Immune Response of Brazilian Children to a *Neisseria meningitidis* Serogroup B Outer Membrane Protein Vaccine: Comparison with Efficacy

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Since 1986, serogroup B *Neisseria meningitidis* has caused approximately 80% of the meningococcal disease in Brazil. In 1988, an epidemic caused by *N. meningitidis* B:4:P1.15 was recognized in the greater São Paulo area of Brazil. The São Paulo state government decided to vaccinate children from 3 to 83 months of age with a vaccine consisting of serotype 4 outer membrane protein and group C meningococcal polysaccharide that was produced in Cuba. About 2.7 million children were vaccinated during two immunization campaigns conducted in 1989 and 1990. Because of this, a case-control study was designed to determine vaccine efficacy against group B meningococcal disease. The purpose of our study was to compare the antibody response with the protection from disease estimated from the case-control study. We measured the immune responses of vaccinees by enzyme-linked immunosorbent assay (ELISA), immunoblot, and bactericidal assay. The development of bactericidal antibodies was age dependent and in good agreement with the results of the case-control study. Only 40% of vaccinees showed fourfold or greater increases in bactericidal antibody titers after vaccination. A poor correlation between antibody levels detected by ELISA and those by bactericidal assay was found. Immunoblot analysis showed that about 50% of the serum samples with bactericidal titers higher than 1:4 were reactive with class 1 outer membrane protein. We conclude that the bactericidal assay is a good, laboratory-based, functional assay for the study of vaccine immunogenicity and that an effective solution to group B meningococcal disease remains to be demonstrated.

Since 1986, serogroup B *Neisseria meningitidis* has been responsible for approximately 80% of the meningococcal disease in Brazil (29). In 1988, an epidemic situation in the greater São Paulo area of Brazil, which extends up to the present, was recognized (6). In 1990, greater São Paulo included 17.8 million inhabitants in 38 municipal regions. A single serogroup B *N. meningitidis* clone, being of the ET-5 complex and having serotype 4:P1.15, has been the prevalent strain in the region (8, 29).

Effective polysaccharide vaccines against *N. meningitidis* serogroups A, C, Y, and W135 are available (10, 15, 17). In contrast, the serogroup B polysaccharide is poorly immunogenic and antibodies to this polysaccharide do not appear to be protective (21). Most of the efforts to develop an effective serogroup B vaccine have therefore focused on lipooligosaccharide-depleted outer membrane proteins (OMPs) (2, 14, 27, 36). Efficacy trials with such vaccines have recently been conducted in Chile, Cuba, and Norway (3, 5, 32). Efficacy levels of approximately 50% were found in the Chile and Norway studies. Better protection was reported for the vaccine produced in Cuba, with an efficacy around 80% against disease caused by a B:4:P1.15 strain (32). This was the first clear demonstration that antibodies induced to noncapsular antigens can protect against meningococcal disease. In view of the epidemic situation in greater São Paulo, i.e., 4.06 cases per 100,000 inhabitants in 1988, and recent acquisition of the Cuban serogroup BC vaccine by the São Paulo state government, it was decided to vaccinate all children from 3 months to 7 years of age. Two immunization campaigns were conducted. In the first (1989), about 300,000 children attending day care centers were vaccinated (12% of the estimated 2.7 million children in the target age range), and in the second (1990), about 2.4 million children (92% of children in the target age range) were vaccinated. Both campaigns were carried out in regional health clinics where children received two doses of the vaccine (6, 7). The vaccination campaigns in São Paulo were the first use of the Cuban-produced vaccine outside of Cuba. Analysis of the immune responses of vaccinated children was considered fundamental to an evaluation of vaccine effectiveness in Brazil. The purpose of this study was to measure the antibody responses of vaccinees by quantitative and functional methods such as enzyme-linked immunosorbent assay (ELISA) and bactericidal assay and to correlate these responses with the protection against disease by using the results of the case-control study (24), which was designed to determine vaccine efficacy against group B meningococcal disease.

MATERIALS AND METHODS

Vaccine. The vaccine was produced in Cuba (it is often referred to as Cuban BC vaccine) from strain Cu385/83 (B:4:P1.15), consisted of lipooligosaccharide-depleted OMPs and group C polysaccharide, and was enriched with envelope proteins from 65 to 95 kDa (32). The group C polysaccharide was present to improve the solubility of the OMPs and to provide protection against group C meningococcal disease. The vaccine was adsorbed onto aluminum hydroxide. Each dose contains 50 μg of group B proteins, 50 μg of C polysac-

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TABLE 1. Antibody levels as measured by ELISA pre- and postvaccination with the Cuban BC vaccine

Age group (mo)	No. of children	Strain of ELISA antigen	Antibody levels (U/ml)[a]		Fold increase	% >Twofold[b]	% >Fourfold[b]
			Prevaccination	Postvaccination			
3–23	275	Cu385/83	25.5	111.2	4.4	81	51
	276	N.150/88	21.7	59.0	2.7	63	35
	120	N.44/89	36.1	156.7	4.3	84	56
	120	N.577/89	37.2	136.3	3.7	85	51
	120	N.614/89	23.9	71.0	3.0	80	42
24–47	144	Cu385/83	23.3	110.5	4.8	85	54
	144	N.150/88	18.6	73.9	4.0	76	45
	20	N.44/89	20.8	97.0	4.7	100	60
	21	N.577/89	20.9	83.9	4.0	90	52
	21	N.614/89	17.0	53.6	3.2	95	38
48–83	141	Cu385/83	27.1	148.0	5.5	87	56
	141	N.150/88	17.6	82.4	4.7	86	56
	19	N.44/89	31.1	173.1	5.6	95	58
	19	N.577/89	23.9	108.9	4.6	89	63
	19	N.614/89	26.3	100.4	3.8	84	58

[a] Geometric mean units per milliliter.
[b] Percentages of vaccinees showing greater than two- and fourfold increases in antibody levels, respectively.

charide, and 2 mg of aluminum hydroxide with 0.01% thimerosal as a preservative (32). Two intramuscular doses of 0.5 ml were administered to children between the ages of 3 and 83 months at an interval of 6 to 8 weeks.

Serum samples from vaccinees. During the first campaign, blood samples were collected before the first dose and 4 weeks after the second vaccination. Serum samples were stored at −20°C. For serological studies, children were classified in the three age groups used for the case-control study. The age groups and numbers of individuals studied can be seen in Tables 1 and 2.

Meningococcal strains. Brazilian strains designated N.44/89 (B:4:P1.15), N.150/88 (B:4:P1.15), N.577/89 (B:4:nt), and N.614/89 (B:NT:P1.15) and a Cuban strain [Cu385/83 (B:4:P1.15)] were used for the preparation of ELISA solid-phase outer membrane antigens. Strains Cu385/83 and N.150/88 were used in immunoblot analysis and as the target strain for the bactericidal assay, respectively. These strains were serogrouped by slide agglutination and serotyped by immunoblot analysis with monoclonal antibodies (29).

ELISA and immunoblot antigens. Cultures of *N. meningitidis* were grown overnight at 36°C in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) on a rotatory shaker at 120 rpm. Outer membrane vesicles (OMV) were prepared by extraction of the wet cell pellet for 2.5 h at 50°C with 5 ml of 0.2 M lithium chloride in a 0.1 M sodium acetate buffer (pH 5.8) per g of cells (33). Protein concentrations were determined by the method of Lowry et al. (22), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the OMV proteins. Sialic acid and 2-keto-3-deoxyoctulosonic acid were analyzed by the resorcinol and thiobarbituric acid techniques, respectively (26, 34). Additionally, strain Cu385/83 was grown in TSB containing 42 μM EDDA [ethylenediamine di(*o*-hydroxyphenylacetic acid); Sigma Chemical Company, St. Louis, Mo.] to chelate free ferric iron and induce the formation of iron-regulated proteins (IRPs). OMVs containing IRPs were used only for immunoblot analysis.

ELISA technique. A standardized ELISA was performed in triplicate in microdilution plates (Difco) as described by Harthug et al. (19) with an alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Sigma) detection system. As an internal antibody standard, a twofold dilution series of a positive postvaccination serum sample was used in all experiments. The mean value of the observed optical density was transformed to arbitrary units per milliliter by a sigmoidal standard curve (log-logit transformation) calculated from the values of the reference serum sample with a computer program provided by Carl E. Frasch. Initially, all serum samples were analyzed at a 1:200 dilution. Samples with optical density values of ≥90% of the maximum optical density of the standard were further diluted and reanalyzed.

Bactericidal assay. A modification of the assay described by Frasch and Robbins was used for determining bactericidal activity (12). Serum samples were titrated in flat-bottom plates (Linbro; Flow) with Hanks' balanced salt solution containing

TABLE 2. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in serum samples from vaccinees

Age group (mo)	No. of children	GMT[a]		% >1:4[b]		% ≥Fourfold increase[c]	% Point estimate of vaccine efficacy (95% CI)[d]
		Prevaccination	Postvaccination	Prevaccination	Postvaccination		
3–23	122	1.1	2.0	2.5	13.1	22	−37 (−100 to 73%)
24–47	44	1.5	4.9	20.4	43.2	45	47 (−72 to 84%)
48–83	44	2.0	9.2	25.0	52.3	52	74 (16 to 92%)
Total	210	1.5	4.6	10.9	36.2	40	54 (Not given)

[a] GMT, geometric mean bactericidal titer.
[b] Percentages of vaccinees with bactericidal titers of greater than 1:4.
[c] Percentages of vaccinees with bactericidal titer increases of at least fourfold.
[d] From the case-control study (24), with the 95% CI also given.

0.1% bovine serum albumin (HBSS-BSA) as diluent. Meningococci were grown to log phase (2 h) in Mueller-Hinton broth (Difco), and then an equal volume of 10% milk (Oxoid) was added. The culture was then aliquoted and frozen at −70°C. An aliquot was thawed and diluted to yield approximately 2,000 CFU/ml, with the final dilution in HBSS-BSA. By this method, a uniform number of organisms could be used for each assay and a comparison with freshly grown cells gave nearly identical results. The source of complement was human serum from a single donor that lacked bactericidal activity against the target strain and by immunoblot showed no reactivity against the class 1 through class 5 OMPs of strain N.44/89. Microtitration plates were incubated at 37°C for 30 min, and approximately 150 μl of Mueller-Hinton agar containing 10% horse serum and VCN inhibitor (BBL Microbiological Systems, Cockeysville, Md.) was added to each well. Plates were further incubated for 24 h at 37°C with 5% CO_2. The CFU per well were determined with the aid of a stereoscopic microscope (×40). The bactericidal titer was determined with the reciprocal serum dilution yielding a ≥50% reduction in the viable count. A positive serum sample and bacterial inoculum plus complement source controls were included in each experiment.

Immunoblot. SDS-PAGE and the detection of antibodies by immunoblot were performed as described by Wedege and Frøholm (35), except that Tris-glycine transfer buffer without methanol was used. Monoclonal antibodies against type 4 (2303C5), subtype P1.15 (2731C6), and class 4 (AE3) and class 5 (AG10) OMPs were used to identify the class 1 to 5 proteins. The monoclonal antibodies 2303C5 and 2731C6 were produced by one of us (C.E.F.), and the monoclonal antibodies AE3 and AG10 were provided by Biomanguinhos Institute, Rio de Janeiro, Brazil. For class 3 monoclonal antibody reaction, Empigen BB 0.25% (Albright and Wilson, Whitehaven, United Kingdom) was added to the primary antibody solution (23). The binding of human IgG and mouse IgG was detected with goat anti-human and anti-mouse IgG antibodies conjugated with horseradish peroxidase. The pre- and postvaccination serum samples of 15 individuals with postvaccination titers greater than 1:4 were tested.

Statistical methods. The ELISA and bactericidal assay results were transformed to logarithmic values to calculate the geometric means. This gave a normal distribution of data and allowed us to use standard statistical tests (1). The significance levels of differences between groups were examined by the Student *t* test, Wilcoxon scores, or the Kruskal-Wallis test on the log-transformed data. Fisher's exact test was used to analyze the differences among percentages.

RESULTS

Antigenic analysis. SDS-PAGE analysis of the antigens used for ELISA showed a predominance of class 1, 3, 4, and 5 OMPs. In strain N.150/88, class 4 and 5 OMPs were present in small amounts (data not shown).

The antigens used for immunoblot analysis can be seen in the first lane of Fig. 1. These nitrocellulose strips were stained with amido black. Class 1, 3, 4, and 5 OMPs predominated for both antigens. Also, some high-molecular-weight IRPs can be seen with antigens from strains grown in TSB with EDDA (Fig. 1B).

For all antigen preparations, the ratio of protein to lipooligosaccharide was approximately 1:1 and only traces of sialic acid could be detected.

Antibody responses to OMPs. Table 1 shows the ELISA antibody levels against different *N. meningitidis* group B strains

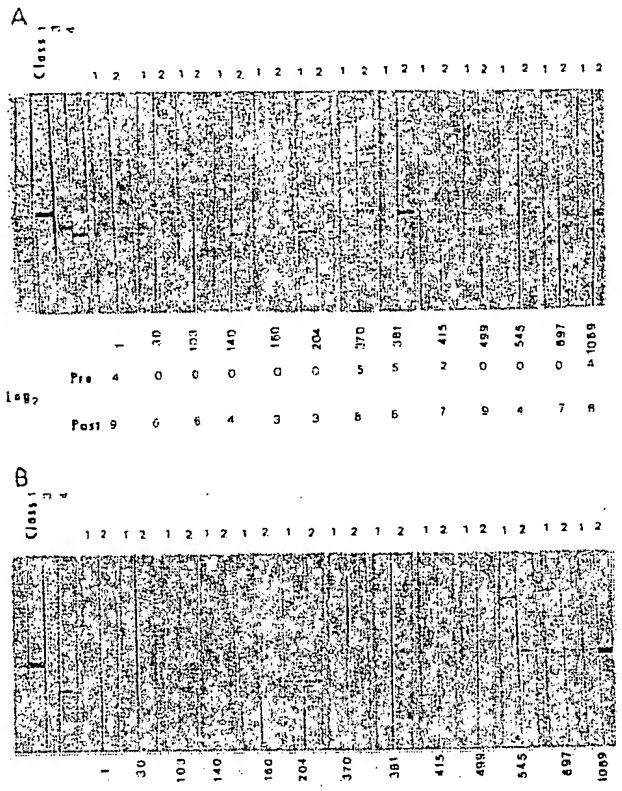

FIG. 1. Immunoblots showing IgG binding to OMPs of *N. meningitidis* Cu385/83 (B:4:P1.15) of pre- (lanes 1) and postimmunization (lanes 2) serum samples from children older than 24 months of age. IgG binding to antigens extracted from the strain grown in normal TSB (A) and TSB containing added EDDA (B) is shown. Amido black-stained strips are shown in the lane at the far left. The next three lanes represent monoclonal antibodies binding to class 1, 3, and 4 proteins, respectively. The bactericidal titers (expressed as $\log_2$ of the reciprocal titers) for pre- and postimmunization serum samples are also shown. The numbers 1, 30, 103, etc., represent vaccinees.

for the age groups examined. The observed differences between the pre- and postimmunization serum samples for all age groups were statistically significant ($P < 0.05$). There were no significant differences in the antibody levels or percent responders among vaccinees among the three age groups studied, except for children from 3 to 23 months of age who showed lower responses against strain N.150/88 ($P < 0.05$). Of all vaccinees studied, 85 and 52% showed at least two- or fourfold increases in antibody levels after vaccination, respectively.

Bactericidal antibodies. Table 2 shows the bactericidal antibody titers against *N. meningitidis* strain N.150/88 (B:4:P1.15) in serum samples obtained before and after vaccination. Some of the serum samples were also tested against two additional B:4:P1.15 strains (N.44/89 and N.131/88), with comparable titers. For each age group, the increase in antibody titers after vaccination was statistically significant ($P < 0.05$). Also, the percentages of children with bactericidal titers of >1:4 pre- and postvaccination are shown. The postimmunization antibody levels in children less than 24 months of age were significantly lower than those of older children ($P < 0.05$). No differences in bactericidal titers or fold increases in children

TABLE 3. Geometric mean bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) in individuals with and without detectable prevaccination bactericidal activity

Age group (mo)	Log 2 bactericidal titer[a]					
	Without activity			With activity		
	Prevaccination[b]	Postvaccination	% ≥1:4[c]	Prevaccination[b]	Postvaccination	% ≥1:4[c]
3–23	0 (76)	1.7	19.7	1.3 (46)	2.5	26.1
24–47	0 (24)	4.3	54.0	2.5 (20)	6.1	45.0
48–83	0 (23)	4.6	43.5	4.6 (21)	19.7	67.0
Total	0 (123)	3.2	39.1	2.5 (29)	6.5	46.0

[a] A value of 1 was assigned to each titer of <1:2; thus, log 2 of 1 = 0.
[b] Values in parentheses are numbers of individuals.
[c] See Table 2, footnote c.

less than 1 year old compared with children 1 to 2 years of age could be seen (data not shown).

The influence of preimmunization antibody levels on bactericidal activity is shown in Table 3. For all age groups, except 24 to 47 months, there were significantly higher bactericidal titers after vaccination for children with demonstrable antibodies before vaccination than for those without such antibodies ($P < 0.05$). No significant differences in the percentages of children showing at least a fourfold increase in antibody titer among the three age groups were found when those with and without previous bactericidal antibodies were compared.

An analysis of the correlation between ELISA and bactericidal assay results is shown in Fig. 2. Poor correlation between the fold increases in antibody levels measured by these two assay techniques was evident. The correlation coefficient (r) varied from 0.2 to 0.4 among the three age groups.

Immunoblot studies. Figure 1 shows the binding profiles of IgG antibodies reactive with different OMPs in the serum samples of 13 vaccinees with bactericidal titers greater than 1:4. The antibodies bound most intensely to class 1, 4, and 5 OMPs. Postvaccination serum samples from individuals 30, 160, 370, 381, 415, 545, and 1069 showed distinct IgG binding to class 1 protein. High-molecular-weight IRPs were recognized in postvaccination serum samples from individuals 30 and 545. Two postvaccination serum samples with bactericidal titers of 1:256 and 1:128 showed no reactions with OMPs (data not shown).

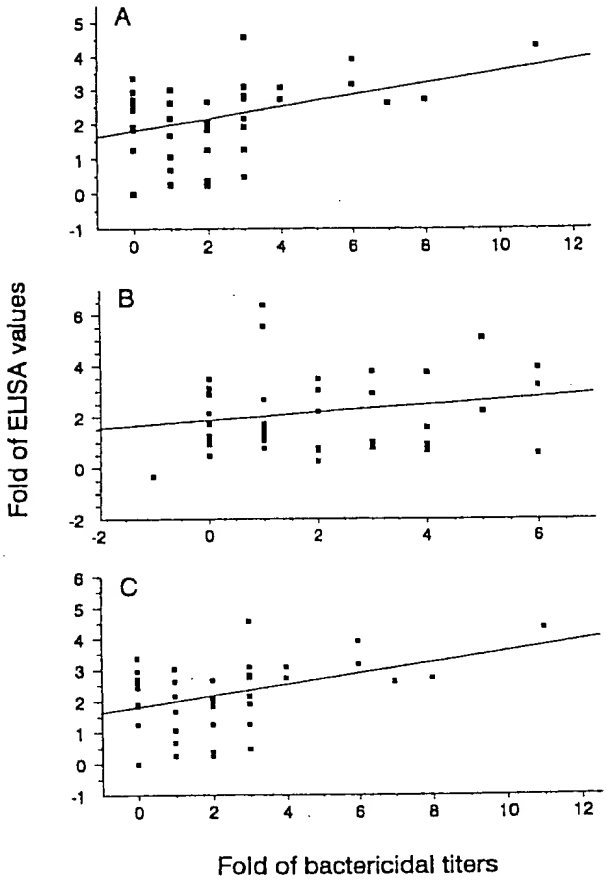

FIG. 2. Correlations between the fold increases of ELISA IgG antibody levels and bactericidal titers against *N. meningitidis* N.150/88 (B:4:P1.15) for children less than 24 months old ($r = 0.20$) (A), from 24 to 47 months old ($r = 0.20$) (B), and from 48 to 83 months old ($r = 0.41$) (C). Least-square regression lines are shown.

DISCUSSION

Bactericidal antibody against meningococcal group C polysaccharide has been shown to correlate with protection against meningococcal disease (10, 16). Some OMPs from group B meningococci induce bactericidal antibodies and have been used as alternative vaccines (4, 11). Several immunologic tests have been used in the evaluation of meningococcal group B protective immunity. These include ELISA studies, serum bactericidal and opsonic assays, and immunoblot studies (18, 19, 28, 35). The vaccination campaign of 2.4 million children from 3 months through 6 years of age in greater São Paulo provided an important and useful opportunity to study the correlation between immunogenicity and vaccine efficacy as estimated by the case-control study.

Our results showed that the functional immune responses of vaccinated children against group B meningococci are age dependent. There were significantly lower bactericidal titers for children less than 24 months of age and also a significantly lower percentage of these children with bactericidal antibodies (titer, ≥1:4) after vaccination compared with the percentages of older children. The percentage of children under 24 months of age with postvaccination titers of greater than 1:4 was only 13% compared with 47.7% for those over 24 months of age. It is interesting to note that the postvaccination seroconversion rate of children under 24 months of age (13%) was less than the naturally acquired levels of those over 24 months of age (Table 2). The age-specific incidence of group B meningococcal disease is much higher in children under 2 years of age, with a peak incidence in children under 1 year old. Our results are similar to those described by Frasch et al. (11), who studied the antibody responses of children to serotype 2a and 2b OMP vaccines combined with group B meningococcal polysaccharide but without adjuvant.

In agreement with our bactericidal assay results, the São Paulo case-control study showed that the estimated vaccine efficacy varied by age. The efficacy was 74% (95% confidence interval [95% CI], 16 to 92%) for children over 4 years old and 47% (95% CI, −72 to 48%) for those 24 to 47 months of age. The vaccine showed no protection for children less than 24 months of age (−37% and a 95% CI of −100 to 73%), and only 13% of the children showed postvaccination bactericidal titers of greater than 1:4 (24).

Greater increases in bactericidal titers after vaccination

were seen for children with preexisting antibodies than for those without such antibodies. Prior exposure to *Neisseria* proteins may explain the more vigorous response to vaccination. It is known that vaccination with a group B meningococcal outer membrane vaccine primes for a later anamnestic response (32). Although the immune responses in subjects without measurable preexisting antibodies were significantly lower than in those with demonstrable antibodies preimmunization, we found similar seroconversion rates as indicated by a minimum of fourfold increases in antibody titers after vaccination. The increase in antibody levels before vaccination was age dependent ($P < 0.05$). These observations support the role of naturally acquired immunity in protection against meningococcal disease.

On the basis of the estimated antibody levels to noncapsular surface antigens as measured by ELISA, the vaccine induced significant immune responses against group B *N. meningitidis* in vaccinees of all age groups. There were, however, some variations in response against the three B:4:P1.15 strains studied. The increase in antibody levels to OMV from strain N.150/88 after vaccination for children less than 24 months of age was significantly smaller ($P < 0.05$) than those for the other two age groups. The OMV from this strain showed smaller amounts of class 4 and 5 proteins in relation to class 1 protein. Perhaps there are important antigenic differences between strains of the same serotype that are isolated in different regions of the country.

The ELISA results with different group B meningococcal serotypes demonstrated more-intense antibody reactivity with 4:nt than with NT:P1.15 OMV. However, no differences in reactivity against these antigens could be seen by immunoblot studies (data not shown). This is not surprising because conformational differences and associations among major OMPs may account for the differences between ELISA and immunoblot reactivities.

The poor correlation between the overall antibody levels to various surface antigens estimated by ELISA and the bactericidal titers can be explained by the fact that the bactericidal assay measures only the subset of ELISA antibodies that are functionally important. Among important factors for bactericidal activity are the isotype and affinity of antibody molecules, as well as the ability of antibody molecules to activate complement (28, 31). Further studies are required to determine the predominant class of immunoglobulins induced by vaccination since they may have different functional activities.

The immunoblot studies showed considerable individual heterogeneity in antibody responses to vaccination. Considering that the primary antigenic epitopes detected by immunoblot are linear epitopes, class 1 and class 4 OMPs appeared to be the most reactive. An important finding was the correlation of IgG antibody binding to class 1 OMP in serum samples with high bactericidal activities (Fig. 1). This was observed for 7 of the 15 serum samples with titers of greater than 1:4. In this regard, others have correlated the antibodies induced to class 1 protein with protection (20, 30). The ability of antibodies to class 4 OMP to block otherwise bactericidal antibodies to specific cell surface antigens has not been well investigated (25). In general, pre- and postimmunization serum samples tested in our study showed weak reactivities with this protein. Serum samples from individuals 140 and 204, whose bactericidal titers postvaccination were 1:16 and 1:8, respectively, were reactive with class 4 OMP. The preimmunization serum sample from individual 204 clearly showed binding to this protein but did not have bactericidal activity. Weak IgG reactivities of 10 serum sample pairs with bactericidal titers of <1:4 were seen on immunoblots (data not shown).

Although the Cuban BC vaccine does not contain increased amounts of IRPs, one of 30 individuals (number 30) showed an increase in IgG binding to IRPs after vaccination. Also, the pre- and postimmunization serum samples from individual 545 showed reactivity with a high-molecular-weight protein. The human immune response to infection indicates that IRPs are expressed and immunogenic in vivo (4). There is considerable interest in exploring the use of one or more of these IRPs as vaccines (2, 9, 13).

These results demonstrate that the bactericidal assay is a good functional assay for the study of group B meningococcal vaccine immunogenicity, which appears to correlate with vaccine-induced protection. It is, nevertheless, also important to include the role of phagocytic killing in protection against meningococcal disease in future studies.

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Serum bactericidal activity correlates with the vaccine efficacy of outer membrane vesicle vaccines against *Neisseria meningitidis* serogroup B disease

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Abstract

For evaluation of serum bactericidal activity (SBA) as surrogate for the efficacy of outer membrane vesicle (OMV) vaccines against *Neisseria meningitidis* serogroup B disease, we have reanalyzed data from a randomized double blind placebo-controlled efficacy trial involving 172,000 secondary school students (aged 13–14 years) in Norway (1988–1991). A cohort of the efficacy trial consisting of 880 individuals was selected for immunogenicity studies. An efficacy of 87% was calculated for a 10-month observation period. However, after an observation period of 29 months, the estimated efficacy against group B disease induced by vaccination was 57%. The immunogenicity study showed that the SBA geometric mean titer (GMT) for the vaccinees was 2.4 before vaccination and 19.0 six weeks after the second vaccine dose. One year after vaccination the GMT was reduced to 2.8. A separate three-dose study with 304 adolescents showed that with a third dose at 10 months after the second dose (i.e. when cases of disease started to appear) a strong booster response was induced. Ten months after the second dose the SBA was reduced to near pre-immunization level. Following the third dose the SBA geometric mean titer of 2.7 increased to 62.3. One year after the third dose, the GMT was markedly higher than 6 weeks after the second dose (12.6 versus 8.8). Thus, protection after vaccination corresponds with the level of SBA. In order to reach lasting protective levels of SBA in a population, three vaccine doses are probably required. Measurements of SBA are likely to be useful for evaluating various upcoming formulations and improvements of immunization regimens for OMV vaccines.

Keywords: Serum bactericidal activity (SBA); Outer membrane vesicle (OMV); Geometric mean titer (GMT)

1. Introduction

In the UK the serum bactericidal activity (SBA) is considered sufficient for licensure of meningococcal conjugate vaccines against serogroup C disease without the classic randomized efficacy trial [1]. The rationale for this decision is based on a long history of empiric observations and laboratory studies. Already in 1918 Matsunami and Kolmer [2] used a whole-blood bactericidal assay to show that the bactericidal activity of the blood of various animals was related to the resistance to infection with *Neisseria meningitidis* of these animals. They also provided the first evidence of age-dependent rise in immunity to meningococci in humans, demonstrating less bactericidal activity of serum from children compared to the activity of sera from adults. Early in the 1920s, Heist et al. showed that blood from more than 95% of adults was bactericidal to meningococcal carrier isolates [3]. This evidence for individual variation in immune responses and individual susceptibility to infection led Heist to postulate that most cases of meningitis occur among those few individuals who lack bactericidal activity [4].

The importance of bactericidal antibodies for protection against meningococcal disease has been demonstrated indirectly by the inverse relation between anti-meningococcal antibody titers or serum bactericidal activity and the age-related incidence of meningococcal disease [5]. Also the observation that individuals with defects in the terminal complement pathway are highly susceptible to meningococcal disease indicates that complement-dependent killing of the bacteria is correlated with protection [6]. More directly the correlation between SBA titers and protection (or rather clinical estimated efficacy) has been shown in various vaccine trials. Most clearly Goldschneider et al. [5,7] and Gotschlich et al. [8,9] showed this for the group C polysaccharide vaccine.

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2. Materials and methods

2.1. Vaccine and vaccinees

The vaccine, vaccinees and immunization schedule for the efficacy trial have been described previously [10,11]. Briefly, the immunogen was deoxycholate-extracted outer membrane vesicles (OMVs) from meningococcal strain 44/76 (B:15:P1.7,16:L3,7,9). The OMVs were adsorbed to aluminum hydroxide, and each dose contained 25 μg of protein. The placebo was $Al(OH)_3$ in 0.5 ml buffer. The vaccine, MenBvac, or placebo was injected as 0.5 ml into the deltoid muscle. *The efficacy trial*, lasting from 1988 until 1991 (29 months), included 172,000 secondary school students (aged 13–14 years), receiving two doses 6 weeks apart. The randomization (1:1) was done at school level. The corresponding *two-dose immunogenicity study* included 880 individuals, a cohort of the exact same group as those participating in the efficacy trial. The randomization in this trial was done on individual level in the proportion vaccine:placebo, 2:1. A separate *three-dose immunogenicity study*, giving two doses 6 weeks apart and a third dose 10 months after the second injection, was done in the same age group from 1993 to 1995. In this study, 311 received MenBvac and 62 in the control group received Meningovax A + C from "Pasteur Merieux". The Norwegian Medicines Control Authority and the Regional Committee for Medical Research Ethics in Norway approved all three studies.

2.2. Serum bactericidal activity

The standardized meningococcal variant 44/76-SL (B:15:P1.7,16:L3,7,9), was used as a target strain in the bactericidal test. Other strains, e.g. for analyzing the heterologous immune response, might be used after appropriate characterization. This test for functional immunity is based on two-fold dilution steps of serum in microplates using the agar overlay method, as previously described [12,13]. The bacterial inoculum was adjusted to 70–90 colony-forming units per well, and the assay was performed in presence of 25% human plasma as an exogenous complement source. The mixture of diluted test serum, bacteria and complement serum was incubated for 60 min (alternatively 30 min; see Sections 3 and 4) at 37 °C before the agar was added and the incubation continued overnight at 37 °C in a 5% CO_2 atmosphere. The titers are given as the reciprocal of the last serum dilution giving >50% killing of the inoculum.

3. Results

3.1. Efficacy trial for MenBvac

The total observation period, for the efficacy trial, was 29 months and the estimated efficacy was calculated to 57% (95% CI = 21–87%). Nearly all cases among vaccinees appeared later than 10 months after immunization (except for one case which occurred after 6 months). In the placebo group, however, seven cases appeared within the first 10 months of the study (Fig. 1). Retrospective calculations at various time points show that the observed protection rate decreased with the increase of time after vaccination. During the first 10 months the estimated efficacy was 87% (95% CI = 62–100%), whereas it was as low as 30% for the last 10 months [14] (see Table 1 and the right part of Fig. 1).

Table 1
The efficacy trial: number of cases and estimated efficacy at different time-points after two doses of MenBvac[a]

Observation time	Number of cases		Estimated efficacy (%)
	Vaccine	Placebo	
0–2 weeks	0	(2)[b]	–
0.5–10 months	1	7	87
11–20 months	4	9	59
21–29 months	6	8	30
0.5–29 months[c]	11	24	57

[a] Vaccine group: 690 schools (89,000 students); placebo group: 645 schools (83,000 students). Total observation period was 29 months.
[b] Two cases of MenB disease occurred in the placebo group during the first 2 weeks. According to the protocol, these two cases were not included in the efficacy calculation.
[c] [11].

3.2. Immunogenicity following two doses

As can be seen from Table 2, the increase in SBA 6 weeks after the second dose, dropped rapidly after the second dose. One year after the second dose the functional immunity was almost reduced to pre-vaccination level (in contrast to antibodies measured in ELISA that persisted at substantial levels up to 52 weeks [15]). After 2 years the SBA level increased somewhat, probably due to natural immunization.

3.3. Immunogenicity following three doses

Data from this study can be seen in Table 3 and the immunogenicity part of Fig. 1. In this study the pre-immunization titers appeared to be lower than in the protection trial. However, this is perhaps due to 30 min incubation time as opposed to 60 min (now used as standard). The latter is thought to increase the sensitivity. Six weeks

Table 2
The two-dose immunogenicity study: SBA in a cohort of the efficacy trial

Time	GMT	Percentage with SBA titer ≥4	Percentage with ≥4-fold rise in SBA
Before first dose	2.4	31	–
6 weeks after first dose	8.5	72	46
6 weeks after second dose	19.0	97	80
1 year after second dose	2.8	32	19
2 years after second dose	3.5	42	26

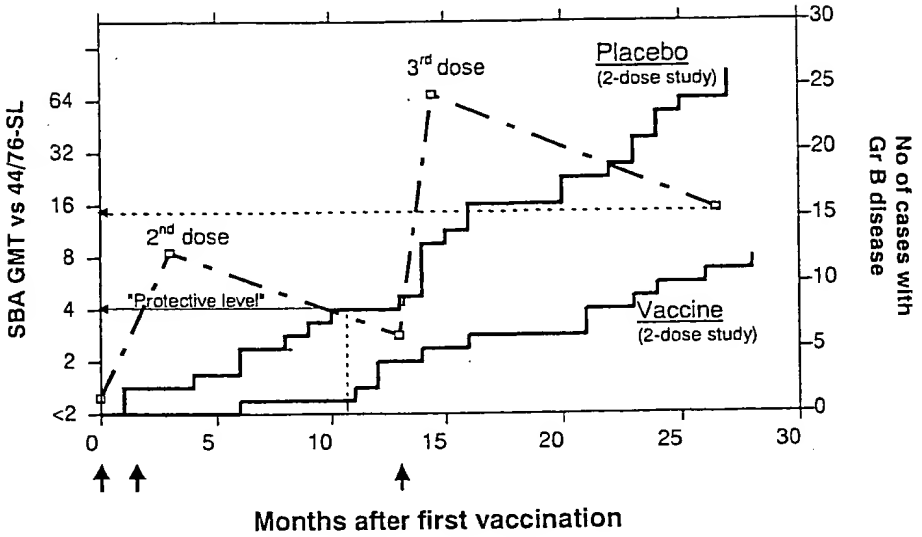

Fig. 1. Bactericidal activity in a three-dose study (left *Y*-axis) as compared to number of cases (cumulative) in vaccine and placebo groups in the original two-dose efficacy trial (right *Y*-axis). All cases with systemic group B meningococcal disease, occurring from week 0 are included.

Table 3
The three-dose immunogenicity study: SBA responses[a]

Time	GMT	Percentage with SBA titer ≥4	Percentage with ≥4-fold rise in SBA
Before first dose	1.2	4	–
6 weeks after second dose	8.8	77	73
10 months after second dose	2.7	35	28
6 weeks after third dose	62.3	96	96
1 year after third dose	12.6	85	81

[a] A 30-min incubation was used. This serology was done prior to the "reanalysis" of the cohort from the efficacy trial. At present 60 min is chosen as standard (see Sections 2 and 3).

after the second dose the SBA level reached a similar height as seen in the two-dose study. The same, rather rapid decline in SBA after two doses was seen in both studies. Ten months after the second dose the geometric mean titer (GMT) was reduced to 2.7. Six weeks after the third dose, however, the GMT increased to 62.3, and one year after this third dose it was 12.6 (more than five times above the level, one year after two doses).

4. Discussion

The immunogenicity measured by the SBA test following two doses of MenBvac reflects what is seen in the efficacy trial: a rather short duration of protective immunity. A third dose at 10 months after the second gave a markedly increase in SBA, which persisted higher than after two doses, even a year after. Fig. 1 is a condensed illustration of key performance-data for MenBvac, with the mean SBA titers of the vaccinees in the three-dose study. It is important to note, that the first part of Fig. 1, i.e. immunokinetics following the first two doses, is similar to the results found in the cohort of the efficacy study (two doses). The immunogenicity data from the three-dose study is compared to the cumulated number of cases appearing with time in the original two-dose efficacy trial. The graph illustrates the point that MenBvac was highly protective up to about 9 months after the second dose. At this time the SBA GMT for the vaccinated population was estimated to be about 4. Ten months after the second vaccination the GMT decreased to 2.7 and "vaccine failures" started to appear. Related to the experimental conditions used, a "mean protective SBA titer" of 4 can be suggested. This value corresponds well with the assumed protective SBA titer for serogroup C disease (8 when baby rabbit complement is used [1]). Furthermore, it can be seen from the figure that a third dose given 10 months after the second dose induced higher and longer-lasting SBA levels. Even 1 year after the third dose, the GMT was about four times higher than the assumed "protective level", which is likely to indicate an increased protection in the vaccinated population.

Bactericidal antibodies against meningococcal group C polysaccharide have been shown to correlate with protection against meningococcal disease [5,16]. For protection against serogroup B meningococcal disease, less clear and general statements have been given (i.e. for all strains, versions of SBA tests and different MenB vaccines [16]). However, for MenBvac all data available show a consistent good correlation between SBA and efficacy. Two comparative international studies, involving the Norwegian vaccine as one of the products tested, have also been consistent with this [17–19].

The SBA test is a biological assay, which may be variable [12,20]. Thus, standardization is of substantial importance. Several factors can influence the SBA titers. These include the choice of bacterial strain, growth conditions of the bacteria, the time of incubation with serum and the source of exogenous complement (human serum or sera from heterologous species such as rabbits or cows). Exogenous

complement from a person with no SBA antibodies and normal complement activity is the procedure chosen as standard. The use of e.g. baby rabbit complement gives substantially higher titers [1].

The target strain in SBA for evaluation of vaccine response of MenBvac is a standardized version of the seed-lot strain 44/76-SL. Among the different technical variables mentioned above, the presence or the degree of expression for some key antigens is most important. One of the key variable outer membrane antigens is the Opc protein (some others are capsule and degree of LPS sialylation) [21]. Human antibodies to Opc are bactericidal, but only bacteria expressing high amounts of Opc are killed in the bactericidal test. The level of surface expressed Opc protein may vary by transcriptional regulation of the *opc* gene, e.g. due to variable growth conditions, and the observed titers may differ depending on the expressed amounts of Opc [12,21]. This illustrates that it is crucial to standardize the inoculum for the target strain in SBA and to include control sera when performing the test. Using defined conditions, the SBA results have proven to be reproducible in both inter- and intra-laboratory studies [17–19].

Results presented in this report demonstrate that the bactericidal test performed according to standardized conditions is a good functional assay for immunogenicity studies of group B meningococcal vaccine. Based on the evaluation of data from three clinical studies with the MenBvac, we conclude that protection after vaccination corresponds with SBA, suggesting that for this vaccine and its improved formulations the SBA test is a good surrogate assay for protection. In order to reach lasting protective levels of SBA, three doses of MenBvac are required.

For different epidemiological situations and other vaccine formulations the SBA test might also be a valid surrogate for protection. However, this needs to be evaluated case by case. The procedure for establishing such a surrogate assay should be through a panel of sera from vaccinees receiving the actual formulation, thorough selection of a suitable target strain and standardized conditions for cultivation of the inoculum and performance of the SBA test.

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Exhibit 5

Serologic correlates of protection for evaluating the response to meningococcal vaccines

Paul Balmer and Ray Borrow†

Meningococci cause serious disease worldwide and the organism remains the most common cause of bacterial meningitis in children and young adults. The only effective means of controlling disease is through vaccination. Although polysaccharide vaccines have been available for serogroup A, C, Y and W135 for many years, serogroup C polysaccharide–protein conjugate vaccines have only recently been licensed in many countries. Conjugate vaccines for combinations of serogroup A, C, Y and W135 are progressing through clinical trials and major efforts are being made to develop a safe and efficacious vaccine against serogroup B. To assess the quality of the immune response after vaccination, laboratory correlates of protection are needed. For serogroups A and C, serum bactericidal antibody is a well established predictor for protection but for serogroup B, other mechanisms besides serum bactericidal antibody may also be involved in conferring protection against disease. The serologic correlates of protection for evaluating the response to meningococcal vaccines are described in this review.

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vaccine
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Meningococcal disease remains a significant global public health problem with approximately 1.2 million cases per year causing an estimated 135,000 deaths [1]. Large epidemics occur in sub-Saharan Africa, in a region known as the 'Meningitis Belt' which extends from Senegal to Ethiopia. *Neisseria meningitidis* serogroup A is mainly responsible for the epidemics observed in this region, however in 2002, a large outbreak of serogroup W135 occurred in Burkino Faso. Endemic meningococcal disease due mainly to serogroups B and C is observed in Europe and the USA, with the addition of serogroup Y contributing to a significant proportion of meningococcal disease in the USA. An epidemic of meningococcal serogroup B disease is ongoing in New Zealand and previous epidemics of serogroup B disease have occurred in Brazil, Norway, Chile and Cuba.

The epidemiology of meningococcal disease in Europe is changing to predominantly serogroup B infection due to a decline in serogroup C infection following the introduction of serogroup C conjugate (MCC) vaccines. Meningococcal serogroup A and C polysaccharide vaccines have been available since the 1970s but have limited use due to the serogroup C components' lack of immunogencity in children aged less than 2 years and the nature of the T-cell-independent immune response stimulated by vaccination which in general fails to induce immunologic memory. Conjugation of the capsular polysaccharide to a protein carrier converts the vaccine to a T-cell-dependent immunogen and is immunogenic in young children [2–7]. Unfortunately no such vaccines exist for serogroup B disease due to the serogroup B capsule being poorly immunogenic [8] and concerns over safety with the possible induction of autoantibodies [9]. Therefore, the approach taken has been to utilize subcapsular antigen either as outer membrance vesicles (OMVs) [10–12], an aggregrate of outer membrane proteins [13] or more recently individual antigens are being explored [14,15].

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This review details what is known to date about serologic correlates of short-term protection for evaluating meningococcal vaccines. The correlates described below are to date only for short-term efficacy following vaccination. It should be noted that even the performance of efficacy trials, such as the one recently performed for Prevnar® (Wyeth, NJ, USA) in the Kaiser Permenente medical center (CA, USA) [16] only provide information on correlates of short-term protection, in this case for the particular pneumococcal conjugate vaccine. Only post-licensure surveillance can help to provide information on long-term efficacy and the immunologic mechanisms correlated with this. An example of this is *Haemophilus influenzae* type b (Hib) disease in the UK where 10 years following the introduction of the Hib conjugate vaccine, long-term efficacy data are available through post-licensure surveillance. Thus, for MCC vaccines described below we are still many years away from realizing the correlates for long-term protection.

MCC vaccines

The UK was the first country to introduce MCC vaccines [17]. A phased introduction commenced in 1999/2000 with infants receiving three doses at 2, 3 and 4 months of age, infants 5 to 11 months of age received two doses and those aged 1 to 19 years received one dose. The vaccination program was extended in 2001 to include those aged up to 24 years [18]. An enhanced surveillance program was initiated [101] which was designed to investigate any vaccine failures and provide vaccination coverage data. Those data generated have been used to calculate vaccine efficacy estimates and to determine if herd immunity was occurring in unvaccinated individuals.

The impact of MCC vaccines has been significant [17,19–20]. A reduction of 67% in the attack rate in the targeted age groups from 2001–2002 was observed with vaccine efficacy estimates at 94% overall [20]. The success of the vaccination program has resulted in a significant reduction in deaths due to meningococcal serogroup C infection from 84 in 1998/1999 in those aged less than 20 years to nine in 2001/2002. The prevalence of nasopharyngeal carriage of serogroup C meningococci has also reduced by 67% in teenagers following immunization with MCC vaccines [21]. Evidence of a herd immunity effect from the MCC vaccines has been recently published demonstrating a 67% reduction in the attack rate in unvaccinated children within the targeted age groups [20].

The success of the MCC vaccines in the UK has also been observed in other European countries, such as Ireland and the Netherlands and has prompted countries, such as Spain, Australia and Iceland to implement similar vaccination programs.

Meningococcal serogroup C correlates of protection

The MCC vaccines were licensed in the UK and elsewhere without large-scale Phase III efficacy trials. Extensive pre-licensure studies funded by both the Department of Health and vaccine manufacturers demonstrated the safety and immunogenicity of the candidate MCC vaccines in the targeted age groups [17,19]. The MCC vaccines were then licensed using those data generated from these studies and the known efficacy and correlates of protection of the meningococcal plain polysaccharide vaccine in children aged 2 years and above. The immunogenicity studies required a substantial effort to develop and standardize laboratory assays to provide data on relevant laboratory correlates of protection.

The original correlate of protection for meningococcal serogroup C (MenC) disease was established by Goldschneider and colleagues [22] as a serum bactericidal antibody (SBA) titer with human complement (hSBA) of ≥ 4 for a short-term of duration (approximately 8 weeks). The study involved bleeding army recruits at the initiation of their basic training and the level of SBA determined in those who subsequently acquired a meningococcal infection during the subsequent 8 weeks. It was found that only three out of the 54 prospective meningococcal cases had circulating SBA titers ≥ 4 prior to initiation of training compared with 444 of 540 controls (those who did not acquire a meningococcal infection) who had a SBA titer of ≥ 4. The correlate of an SBA titer was then used to determine the relationship between the age-related incidence of disease and the presence of bactericidal antibody above the proposed cut-off. Sera from children and military recruits were used to give an age range from 0–26 years. The percentage of individuals with a SBA titer ≥ 4 in defined age groups was observed to have an inverse relationship to the incidence of MenC disease. The low incidence of MenC disease in early infancy correlates with a high proportion of individuals with an SBA titer ≥ 4 due to maternal antibody. As the percentage with a SBA titer ≥ 4 declines from birth, the incidence of MenC disease increases peaking at 1–2 years. From 2 years onwards, except for a small peak in young adults, the incidence of disease is lower, while the percentage with an SBA titer ≥ 4 remains high. The study by Goldschneider and colleagues established that an SBA titer of ≥ 4 was an appropriate individual correlate of protection but also that this translates to a correlate for a population. An interesting observation made in the studies performed by Goldschneider and colleagues was that the bactericidal activity observed was not limited to capsular polysaccharide-specific antibodies because sera from army recruits demonstrated bactericidal activity against serogroups A, B and C [22]. Further work confirmed that antibodies to subcapsular antigens were of importance by the observation that adsorption of sera with serogroup C polysaccharide inhibited bactericidal activity to serogroup C strains but not to serogroups A and B [23]. Hence, it is likely that the correlate of short-term protection established as an SBA titer ≥ 4 included capsular polysaccharide and subcapsular antigen specific bactericidal activity. However, Goldschneider *et al.* [22] also stated that although an SBA titer of ≥ 4 indicates protection, an SBA titer of <4 did not always indicate susceptibility to MenC disease.

The classical studies performed by Goldschneider and colleagues established SBA as the 'gold standard' correlate of protection. However, this was achieved using human complement as the source of exogenous complement in the bactericidal assay which is difficult to obtain and standardize,

thus preventing significant inter-laboratory comparisons of data. It is now recommended that commercially available baby rabbit complement is used in a standardized bactericidal assay (rSBA) for evaluation of meningococcal polysaccharide vaccines [24]. The immunogenicity data from the evaluation studies of the MCC vaccines in the UK, was generated using baby rabbit complement. Meningococci are known to be more susceptible to complement-mediated lysis in the presence of exogenous rabbit complement compared with human complement [25–26]. Thus, in the UK, two approaches were undertaken to re-evaluate the SBA and arrive at a protective titer for the rSBA.

First, the inverse relationship between disease incidence and SBA was recently re-assessed in the UK using the rSBA [27]. A total of 1689 sera from age-stratified individuals (ranging from 53–267 per age group), collected prior to the introduction of the MCC vaccine (1996–1999) were tested and SBA titers plotted against disease incidence for those age groups (FIGURE 1). Similar plots to the Goldschneider and colleagues study [22] were observed with peak incidence of disease occurring in infants and young children and a secondary peak occurring in older teenagers. It is interesting to note that the percentage of infants with a rSBA titer ≥ 8 that corresponds to the peak incidence of disease in the UK data is similar to the percentage of US infants with a hSBA titer ≥ 4 in the studies of Goldschneider and colleagues [22]. There are important differences between the two studies, such as the sample size, which was substantially bigger in the UK study and the epidemiology of the study populations that may account for the differences in magnitude of responses especially in the older age groups. The inverse relationship between disease incidence and the prevalence of protective antibody titers as described previously [22] appears more consistent with a rSBA titer ≥ 8 than ≥ 128 (FIGURE 1). This study provided evidence for the establishment of a correlate of protection for MenC disease using rSBA titers rather than hSBA.

However, it was unknown if the established correlate would be appropriate for responses to conjugate vaccines, hence the SBA correlate of protection required further evaluation. An rSBA titer < 8 or ≥ 128 was found to discriminate susceptibility and protection, respectively when compared with the human protective SBA titer ≥ 4 [28]. It was proposed that for those individuals with an rSBA titer between 8–64, additional evidence was required, such as a hSBA titer ≥ 4, a fourfold rise in SBA titer from pre to postvaccination, evidence of antibody avidity maturation or a typical booster response to a polysaccharide challenge [28].

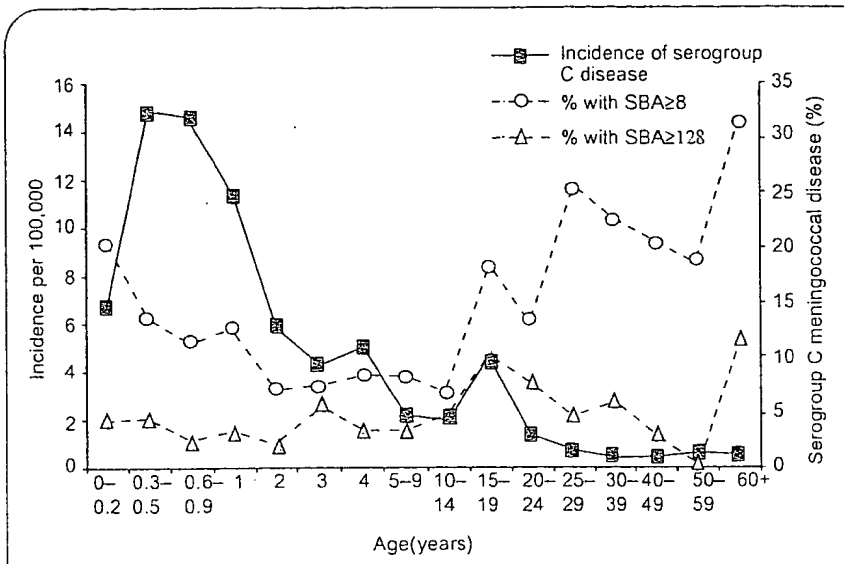

Figure 1. Age-specific incidence of laboratory confirmed serogroup C meningococcal disease in England and Wales 1997/98 and the prevalence of SBA titers ≥8 and ≥128 [22].

Establishment of an individually-based correlate of protection using rSBA titers would be extremely difficult and require a large cohort (approximately 250,000) to be analyzed postvaccination and followed up for subsequent development of disease [29]. Hence, it is deemed more appropriate to rely upon a population-based correlate which may be extrapolated to give a threshold required to protect an individual from disease [30]. The post-licensure surveillance data generated in the UK has enabled vaccine efficacy estimates to be calculated. Hence, to establish a population-based correlate of protection, the level of rSBA titer achieved by the majority of a vaccinated group and not achieved by the majority of an unvaccinated group can be compared with the vaccine efficacy in a specific age group. An rSBA of ≥ 8 was found to give strong correlation with the vaccine efficacy estimate for toddlers, 1 month postvaccination indicating this is an appropriate correlate of short-term protection. At 7 months postvaccination, all rSBA titers underestimate the vaccine efficacy observed in the toddler age group suggesting that the individuals are at least reliant upon immunologic memory. The differences between the Goldschneider approach and the UK approach are summarized in TABLE 1.

Long-term protection following MCC vaccination is assumed to be conferred by the induction of immunologic memory as it is known that in infants and toddlers antibody levels decline rapidly [31]. The MCC vaccines have been shown to induce memory responses in all targeted age groups including young children [3–5]. The traditional method to demonstrate the presence of memory B-cells is to challenge with a plain polysaccharide and observe if a typical booster response is present. However, an alternative laboratory surrogate has been developed which can be used to demonstrate that those

Table 1. Serologic correlation options for meningococcal serogroup C disease.

	Goldschneider *et al* (1966 to 1968)	UK (1999 to 2001)
Population	Military recruits	Toddlers (1–2 years)
Number	14,744	Approx 450,000
Intervention	None	Conjugate vaccine
Observation Period	8 weeks	Average 9 months (range 1–18 months)
Serum bactericidal assay	Human complement	Rabbit complement
SBA threshold	1:4	Range
Method	Prospective cohort study	Observational study (post-licensure surveillance)
Sera	Not available	available

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individuals with low levels of circulating SBA have been successfully primed and would be able to mount a rapid memory response if challenged. The generation of immunologic memory requires affinity maturation of humoral responses to T-cell-dependent antigens. This occurs in germinal centers where B-cells undergo somatic mutation, the random mutation of the immunoglobulin (Ig) variable region genes producing B-cell receptors of high affinity for antigen. Competition for survival signals occurs within the germinal center between B-cells of differing affinities with the selection process favoring those with high affinity receptors. Failure to obtain the correct survival signals results in apoptosis. The selected B-cells, following a cognate interaction with T-cells, leave the germinal center and form either memory cells or plasma cells that produce antibodies of a high avidity.

The determination of antibody avidity indices has been utilized as an indicator of affinity maturation and the induction of immunologic memory [3,5,31–33]. Avidity indices have been used to illustrate the induction of memory following MCC vaccination [3,5] and have shown to increase in the presence of declining circulating SBA [5,31]. In children immunized with MCC vaccine 4 years previously, SBA titers had declined to prevaccination levels whereas avidity indices increased following the primary vaccination and remained constant [31]. Following a challenge with plain polysaccharide vaccine, a typical booster response was observed, characterized by the production of high avidity antibody. Hence, determination of avidity indices can be used to assess if successful priming of memory responses have occurred following conjugate vaccination. It seems possible therefore, that it may be necessary to use SBA titer as a correlate of short-term protection and assessment of immunologic memory as a predictor of longer term immunity.

Determination of avidity indices following conjugate vaccination may only be appropriate in children. Adults tend to produce an antibody response of higher avidity, naturally and even following polysaccharide vaccination [34]. Absence of a maturation of antibody avidity has been observed in adults following immunization with pneumococcal plain polysaccharide or conjugate vaccines [35–36]. As shown by Goldschneider and colleagues [22] and more recently, Trotter and colleagues [27], natural immunity to meningococcal disease in adults was associated with a low incidence of disease. It is thought that natural exposure to meningococci or exposure to cross-reactive antigens throughout life results in the development of memory B-cells and that any challenge encountered, such as polysaccharide or conjugate vaccination will stimulate the memory B-cells present. Hence, the use of avidity indices may be limited to the assessment of immunologic priming of young children. However, it is important to note that unlike the correlate of short-term protection, SBA titer, which has been validated using postlicensure efficacy estimates in the UK, no thresholds have been defined for avidity indices.

An alternative method to avidity indices is being investigated in our laboratory. Throughout a primary course of vaccination the level of both SBA titer and serogroup-specific IgG increase. Affinity maturation is reflected by an increase in antibody avidity which is likely to result in a greater proportion of the antibody produced in response to vaccination to be functional i.e., bactericidal. The SBA detected tends to be of high avidity whereas the serogroup-specific IgG is both high and low avidity antibody. Hence, the ratio of SBA titer to serogroup-specific IgG may be a useful method of detecting the induction of memory following vaccination. This approach has been used previously to assess the response to serogroup B vaccines containing outer membrane complexes [37]. The relative bactericidal activity (RBA: bactericidal titer/outer membrane complex specific IgG) was observed to increase following primary vaccination and continued to increase after a second dose of vaccine reflecting the affinity maturation of the response. Preliminary investigations in toddlers and older age groups suggest that those data generated analyzing SBA titer – serogroup-specific IgG ratio – reflects the induction of immunologic memory as indicated by avidity indices for various age groups following MCC vaccination (UNPUBLISHED DATA). This analytical method requires further validation but it may be applicable for analysis of memory responses to other meningococcal conjugate vaccines with the advantage of removing the need for an additional assay to be performed.

Meningococcal serogroup A correlates of protection

For serogroup A disease, the only correlate of protection we have to date is from the Finnish efficacy trials of meningococcal serogroup A polysaccharide vaccine [38–39] and was determined by radioimmunoassay to be 2 µg/mL. This value was the mean level in unimmunized adults [38].

The SBA assay has not been widely used since the early studies of Goldschneider and colleagues [22] and interestingly, the strain A1 Goldschneider used in these studies is no longer considered optimal as it easily lysed even in prevaccination sera in the bacteri-

cidal assay, therefore tending to very high SBA titers. The Goldschneider studies also used human complement [22]. The serogroup A SBA assay was also used in the Finnish field trials in the 1970s and though the strain used was not quoted, the referenced methodologies eluded to the use of the rSBA [39]. Thus, since the early Goldschneider studies, only rabbit complement has been used in the serogroup A bactericidal assay [40–42]. Most other serogroup A vaccine trials have relied upon solely measuring total serogroup A polysaccharide antibody concentration. With the generation of the rSBA correlate of protection for serogroup C license it is likely that serogroup A conjugate vaccines will be evaluated and licensed on the back of rSBA and appropriate safety data. Extensive trials will be required to confirm the immunogenicity of a candidate serogroup A conjugate vaccine which will provide the opportunity to further validate and standardize laboratory assays, such as SBA and enzyme-linked immunosorbent assay (ELISA).

Immunologic memory responses to meningococcal serogroup A vaccination, both unconjugated and conjugate vaccines, have been evaluated using both the response to a polysaccharide challenge and avidity indices. It is interesting to note that the serogroup A capsular polysaccharide does not appear to be a traditional T-cell-independent antigen as is the serogroup C capsular polysaccharide. First, serogroup A unconjugated vaccine has been shown to be immunogenic in children under 2 years of age [43]. Second, the serogroup A polysaccharide appears to stimulate affinity maturation, as indicated by an increase in avidity indices following vaccination [44]. However, it does appear that there is a difference in the quality of the response induced if the serogroup A polysaccharide is conjugated or unconjugated. A greater level of bactericidal antibody is produced following serogroup A polysaccharide conjugation and the memory response appears to be sustainable [44]. Furthermore, in those aged between 10 and 29 years, serogroup A responses following repeated A/C polysaccharide vaccination were boosted, whereas for serogroup C, immunologic hyporesponsiveness was observed [45].

Meningococcal serogroup B correlates of protection

The poor immunogenicity of the serogroup B capsular polysaccharide means that the conjugation technology applied to vaccines for serogroups A, C, Y and W135 may not be appropriate and alternative vaccine approaches have to be investigated. A number of OMV vaccines have been trialed and utilized to contain epidemics in Norway [10], Brazil [46], Cuba [11] and Chile [47]. Data from the Norwegian serogroup B efficacy trial that took place from 1988–1991 has been recently re-analyzed to evaluate SBA as the appropriate correlate of protection for serogroup B [48]. This randomized double-blind, placebo-controlled efficacy trial involved 172,000 school children aged 13 to 14 years who received two doses of the OMV vaccine (strain B:15:P1.7,16:L3,7,9). The estimated efficacy after 10 months and 29 months was 87 and 57%, respectively. An immunogenicity study using human complement in the SBA, showed that 31% of the vaccinees had SBA titers ≥ 4 before vaccination and 97% reached this SBA titer 6 weeks after the second dose. Titers ≥ 4 persisted in 42% of vaccinees 2 years postvaccination. The percentage of subjects with ≥ fourfold rises in SBA titer 6 weeks post second dose was 80% whilst this percentage decreased to 26% 2 years postvaccination. A separate three dose immunogenicity study was also performed with the cohort receiving a third dose at 10 months. The inclusion of a third dose was observed to improve the clinical performance of the Norwegian OMV vaccine with 96% of individuals achieving an SBA titer ≥ 4 or a ≥ fourfold rise in SBA titer 6 weeks after the third dose [48]. The good response observed persisted to 1 year after the third doses with 85% of individuals with a SBA titer ≥ 4 and 81% with a ≥ fourfold rise in SBA titer. Good correlation between observed protection and SBA titers was found and a tentative protective SBA titer of ≥ 4 was proposed. Thus, measurements of SBA may be used to evaluate different vaccine formulations against serogroup B disease.

Most studies of serogroup B OMV vaccines have not relied on absolute SBA titers as a cut off, but have used ≥ 4-fold rises from pre-to postvaccination. This approach seems more rational as titers can vary in magnitude between different laboratories due to the inherent variation of the SBA assay, yet the percentage with ≥ fourfold increases remains relatively constant [48,49].

Assessment of avidity indices as a marker of antibody maturation following meningococcal OMV vaccination has now been utilized [50,51]. These assays have been of use when investigating avidity maturation in different dose schedules of OMV vaccines and in the study of Longworth and colleagues [50] indicated that avidity maturation was taking place even though SBA titers did not reach putative protective levels in all immunized infants. Further studies using avidity indices as a marker of vaccine responses following OMV vaccination are required to gain full understanding of the usefulness of these assays in predicting both protection and immunologic memory in combination with clinical data.

Additional correlates of protection

SBA titers and assessment of immunologic memory have been generally accepted as appropriate correlates of protection, however, there are other mechanisms of protection involved in immunity to meningococcal disease that might be used as correlates of protection.

The whole blood killing assay is a method that can assess the bactericidal activity of blood incorporating antibody-mediated complement lysis and phagocytosis i.e., both humoral and cellular killing mechanisms [52–54]. It has been suggested that the whole blood assay is more sensitive than the SBA [5,31] with bactericidal activity detected in some individuals by the whole blood assay but not by the SBA assay. However, the whole blood assay is unlikely to be suitable for the evaluation of candidate serogroup B vaccines due to the requirement for large volumes of fresh blood for the assay and the difficulty in standardization of the assay.

Opsonophagocytosis is one mechanism that has been demonstrated to be bactericidal against meningococci [55–57]. *Neisseria meningitidis* can be phagocytosed by polmorphonuclear (PMN) cells [58–60] and monocytes [61,62]. The opsonophagocytic assay (OPA) has provided a useful correlate of protection for

Streptococcus pneumoniae along with the ELISA [63] and could provide additional information regarding protection against *N. meningitidis*. The OPA can be semi-automated and use beads coated with capsular polysaccharide and can also be multiplexed to analyze four serogroups in the one assay [64]. The OPA can also use inactivated bacteria as the target [65] but concerns have been raised that in the treatment of the meningococci, new or hidden epitopes may be exposed that have no functional relevance [66,67]. Furthermore, there are issues of standardization between laboratories, particularly regarding the use of cultured cell lines or freshly obtained PMN cells. Further validation of the OPA is required to determine if it is a suitable correlate of protection. A recent study has suggested that SBA is the main immune effector of the vaccine protection against *N. meningitidis* rather than opsonophagocytosis following serogroup C vaccination [68]. It is possible that it may be more appropriate for the evaluation of serogroup B vaccine candidates where the protective epitopes are subcapsular and may involve a greater role for cell-mediated immunity unlike serogroups A, C, W135 and Y where the capsular polysaccharide is the focus of the protective mechanisms. An OPA to analyze the level of protection against serogroup B isolates have been developed [65,67,69]. A surface labelling technique which detects the initial stage of antibody binding to killed meningococci has also been developed and shows potential as an additional assay for the evaluation of serogroup B vaccines [69].

Another method that has been used to assess immune responses to meningococci is animal models. This approach can provide information on the impact of the host–pathogen relationship on whole tissues and the interaction between both humoral and cellular immune responses. However, there are disadvantages of the animal models, including the fact that the only natural host for *N. meningitidis* is humans and that there are no good models available that mimic the course of human disease.

The murine model has been most extensively used. This involves an intraperitoneal challenge of mice with meningococci but also requires an additional iron source for the infecting bacterial strain [70]. The murine intraperitoneal challenge model has been used to assess active protection and passive protection for evaluation of vaccine candidates [71–73]. This model was recently utilized to demonstrate protection against different serogroups and strains conferred by *Neisseria lactamica* immunization [74]. It is interesting to note that although protection was observed against a lethal challenge, no bactericidal activity was observed in mouse sera.

An infant rat model has also been developed that has the advantage of not requiring an additional iron source and lower inocula are required to produce disease than in the mouse model. However, the rat model does not allow active protection to be assessed, not all strains are virulent and the duration of bacteremia is short and mortality low. It has been used to demonstrate passive protection with antibodies against a number of meningococcal components [75,76] and has been used in evaluating responses to serogroup B meningococci [76–79]. One anomaly is that anticapsular antibody has been shown to be protective against a serogroup B challenge in the infant rat model [80] yet the serogroup B capsular polysaccharide is only poorly immunogenic in humans. Furthermore, the assessment of the protection conferred by a monoclonal antibody specific for inner-core lipopolysaccharide epitope in an infant rat model was observed to correlate with bactericidal and not opsonophagocytic activity [81], illustrating the conflicting data that is currently being generated by animal models. Recently, differences in the response of adults to vaccination with plain polysaccharide serogroup C vaccines or MCC vaccines were investigated using an infant rat model [82]. It was observed that at a limiting dose, the immune sera from vaccinees receiving the MCC vaccine was able to confer a greater degree of protection than sera from those receiving the unconjugated polysaccharide vaccine. The infant rat model has also been used to evaluate the potential vaccine candidate, genome-derived Neisserial antigen 2132 (GNA2132) [83]. Anti-GNA2132 antibody was shown to bind to the surface of serogroups B or C and could passively protect infant rats against meningococcal bacteremia yet lacked bactericidal activity, again highlighting the discrepant data generated between the rat model and SBA assay.

Further data are required to establish both the OPA and animal models as credible correlates of protection. It may be that, as stated, they are more appropriate for use in the evaluation of serogroup B vaccines that are likely to be noncapsular polysaccharide vaccines and induce a wider range of immunologic mechanisms unlike the MCC vaccines which induce mainly bactericidal antibody.

Conclusions

The introduction of the MCC vaccines in the UK has been successful in significantly reducing the level of serogroup C disease incidence. The previously determined correlate of protection, SBA, for natural immunity to serogroup C disease has been re-evaluated for MCC vaccines. An rSBA titer of 8 has been validated using the vaccine efficacy estimates as an appropriate correlate of short-term protection. For long-term, a more appropriate correlate may to be the assessment of immunologic memory or a balance of circulating antibody and memory or even an as yet unrealized mechanism. The majority of data have been generated from the determination of avidity indices, which has been shown to reflect affinity maturation and the induction of immunologic memory in infants and young children following MCC vaccination. It is currently unclear if avidity indices are a valid correlate for older age groups due to the production of antibody with a higher avidity in this age group. Additional correlates of protection, such as opsonophagocytic titer, may be of importance for OMV vaccines.

Expert opinion

The introduction of the MCC vaccines into the UK was backed by extensive safety and immunogenicity studies that provided those data required for licensure. Standardization and optimization of laboratory assays were required to evaluate the immunogenicity of MCC vaccines in various age groups and to provide data on appropriate correlates of protection. A combination of immunogenicity data and information gathered through the

enhanced surveillance program to generate vaccine efficacy estimates, have been used to validate the serum bactericidal assay, and to some degree, avidity indices as correlates of short-term protection. These data have set a precedent for the evaluation of future meningococcal capsular polysaccharide–protein conjugate vaccines that include four serogroups A, C, W-135 and Y, and are likely to be licensed over the coming years. It is expected that these vaccines will induce similar responses, i.e., T-cell-dependent production of SBA, as the MCC vaccines. The serum bactericidal assay for serogroup C has been well characterized but there may be some issues regarding the assays for the remaining serogroups which are not as extensively standardized. Tetravalent plain polysaccharide vaccines have been available for several years and successfully induce protective responses in older age groups but the conjugate vaccine(s) should be immunogenic in younger children.

Unlike the tetravalent conjugate vaccines that will be available in the future and are predicted to be as successful as the MCC vaccines, the prospect of a successful universal serogroup B vaccine for global use is less encouraging. The poor immunogenicity and the potential for autoimmunity due to the structural similarities to fetal neural cells means the use of a conjugated serogroup B polysaccharide vaccine will be difficult. Therefore, different approaches are being taken to vaccine formulation with the assessment of immune responses to surface-exposed proteins and noncapsular polysaccharides essential in identifying candidate antigens. Hence, the different composition of the candidate vaccines dictates that additional protective mechanisms are investigated that may be found to be suitable laboratory correlates of protection for serogroup B vaccines. This will include SBA and opsonophagocytic titers, antibody surface labelling, immunologic memory, T-cell responses and protection in animal models. The most substantial data generated for serogroup B vaccines has been from ' wild type' OMV (wtOMV) vaccines based on one representative epidemic bacterial isolate. However, these wtOMV vaccines, tend to induce functional immunity (SBA) to the homologous (vaccine-type) strain and only low levels (approximately 10%) of response to heterologous strains in infants [47]. In adults, the heterologous SBA responses have been shown to be about 50–60% [47]. Numerous, phenotypically and genotypically diverse serogroup B strains cause invasive disease in the UK (and other countries) and hence it is unlikely that a monovalent vaccine will be suitable. Further work is required to establish correlates of protection for serogroup B to enable the thorough evaluation of the immunogenicity of candidate serogroup B vaccines.

Five-year view

With the recent resurgence of Hib disease and the decline of efficacy in the UK [84], a catch up program of a booster dose of Hib conjugate vaccine commenced in the UK in May 2003 [85,86]. Factors which may have contributed to this increase are first, a waning over time of the impact of the catch-up campaign (1–4 years of age), therefore the rate of Hib disease seen now may reflect the true level of protection given by Hib vaccine as part of the routine childhood program alone. Secondly, a diphtheria–tetanus–acellular pertussis–Hib vaccine used in 2000 and 2001 may not have provided the same high level of protection against Hib disease as the vaccines used before this period, or subsequently. Moreover, as this population of children, immunized with a 3 dose vaccine course were known reliably to induce immune memory [87], this suggests that absolute levels of circulating antibody may be of more importance than previously thought. This may have implications for the MCC vaccine and studies looking at booster doses in preschool children will undoubtedly commence in the near future.

A project to introduce a serogroup A conjugate vaccine into Africa has recently been initiated by the World Health Organization (WHO)/Program for Appropriate Technology for Health (PATH) [88]. The aim of the project is to alleviate the large epidemics of serogroup A disease that occur in the sub-Saharan meningitis belt in Africa. It is hoped that the vaccine will be produced, validated and ready for introduction within the next 5–10 years. Trials followed by introduction of this vaccine in Africa will assist in the establishment of correlates of protection for serogroup A.

Further information is required on immunity to serogroup B disease and new candidate vaccines will require assessing. Currently New Zealand is experiencing an epidemic of serogroup B disease dominated by a single strain (B:4:P1.7–2,4) which commenced in 1991 and has seen the incidence of meningococcal disease rise from 1.6 per 100,000 prior to the epidemic to 13.9 per 100,000 over the period 1996–2000 [89]. Since the epidemic is dominated by a clonal outbreak of a serogroup B meningococci with a single PorA serosubtype, it was decided that a homologous PorA-based OMV vaccine would have a role in controlling the epidemic [89]. A candidate vaccine has been developed and is currently undergoing Phase I and II trials in New Zealand [90]. Serologic analysis is primarily using bactericidal assays and will provide extensive data, which will help to determine if SBA titer is an appropriate correlate of protection for meningococcal serogroup B OMV-based vaccines.

Information resources

Articles recommended for further reading

- Miller E, Salisbury D, Ramsay M. Planning, registration and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. *Vaccine* 20, S58–S67 (2001).
- Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus: I. The role of humoral antibodies. *J. Exp. Med.* 129, 1307–1326 (1969).
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- Andrews, N, Borrow R, Miller E. Validation of serological correlate of protection for meningococcal C conjugate vaccine using efficacy estimates from post-licensure surveillance in England. *Clin. Diag. Lab. Immunol.* 10, 780–786 (2003).

The following websites contain additional information on meningococci & meningococcal disease

- Website of the Health Protection Agency: www.hpa.org.uk Accessed 21st December, 2003.
- Meningitis health topics page on the website of the World Health Organization: www.who.int/health_topics/meningitis Accessed 21st December. 2003.
- Website of the central resource for the Neisseria research community: www.neisseria.org Accessed 20th December, 2003.
- Website of the Meningitis Research Foundation: www.meningitis.org Accessed 20th December, 2003.
- Website of the Meningitis Trust: www.meningitis-trust.org.uk Accessed 21st December, 2003.

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Key issues

- The introduction of meningococcal serogroup C vaccines in the UK has been successful in significantly reducing the incidence of serogroup C disease. There is also evidence of a herd immunity effect reducing the incidence in unvaccinated individuals.
- The established correlate of protection for serogroup C, serum bactericidal antibody (SBA), has been validated against the vaccine efficacy estimates demonstrating that a SBA titer of 8 is an appropriate short-term correlate. For the estimation of long-term protection, the assessment of priming of immunologic memory may be a suitable correlate of protection.
- To evaluate future meningococcal conjugate vaccines, it is accepted that determination of SBA titers and immunologic memory in response to vaccination will be crucial in predicting the protective potential of the actual vaccines.
- Correlates of protection need to be established for immunity to serogroup B meningococci to enable the evaluation of candidate vaccines. This may include not only SBA titers and immunologic memory but also additional protective mechanisms.

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1. Occupational lung disease: asbestosis, byssinosis, silicosis, coal workers' pneumoconiosis, lung cancer, and occupational asthma.

2. Musculoskeletal injuries: disorders of the back, trunk, upper extemity, neck, and lower extremity; and traumatically induced Raynaud's phenomenon.

3. Occupational cancers (other than lung): leukemia; mesothelioma; and cancers of the bladder, nose, and liver.

4. Amputations, fractures: eye loss, lacerations, and traumatic deaths.

5. Cardiovascular diseases: hypertension, coronary artery disease, and acute myocardial infarction.

6. Disorders of reproduction: infertility, spontaneous abortion, and teratogenesis.

7. Neurotoxic disorders: peripheral neuropathy, toxic encephalitis, psychoses and extreme personality changes (exposure-related).

8. Noise-induced loss of hearing.

9. Dermatologic conditions: dermatoses, burns (scalding), chemical burns, and contusions (abrasions).

10. Psychologic disorders: neuroses, personality disorders, alcoholism, drug dependency.

11. Control technology research: application of scientific principles to control strategies, preconstruction review, technology forcing/new source performance concepts, technology transfer, substitution, unit operations approaches.

12. Respirator research: new and innovative respiratory protective devices; techniques to predict performance; effectiveness of respirator programs; physiologic and ergonomic factors; medical surveillance strategies; psychological and motivational aspects; and physical properties.

FOR FURTHER INFORMATION CONTACT:

For technical information: Roy M. Fleming, Sc.D., Associate Director for Grants, NIOSH, Building 1, Room 3053, Centers for Disease Control, Atlanta, GA 30333, Telephone: 404/329-3343 or FTS 236-3343

For business information: Leo A. Sanders, Chief, Grants Management Branch, Procurement and Grants Office, Centers for Disease Control, 255 E. Paces Ferry Road, NE. Room 321, Atlanta, Georgia 30305. Telephone: (404) 262-6575 or FTS 236-6572.

(This program is described in the Catalog of Federal Domestic Assistance Program No. 13.262, Occupational Safety and Health Research Grants)

Dated: August 13, 1985.

Elliott S. Harris,

Acting Director, National Institute for Occupational Safety and Health.

[FR Doc. 85-19946 Filed 8-20-85; 8:45 am]

BILLING CODE 4160-19-M

Food and Drug Administration

Advisory Committee Meeting; Filing of Annual Reports

AGENCY: Food and Drug Administration.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing that, as required by the Federal Advisory Committee Act, the agency has filed with the Library of Congress the annual reports of those FDA advisory committees that held closed meetings.

ADDRESS: Copies are available from the Dockets Management Branch (HFA-305), Food and Drug Administration, Rm. 4-62, 5600 Fishers Lane, Rockville, MD 20857, 301-443-1751.

FOR FURTHER INFORMATION CONTACT: Richard L. Schmidt, Committee Management Office (HFA-306), Food and Drug Administration, 5600 Fishers Lane, Rockville, Md 20857, 301-443-2765.

SUPPLEMENTARY INFORMATION: Under section 13 of the Federal Advisory Committee Act (Pub. L. 92-463, 86 Stat. 770-776 (5 U.S.C. App. I)), FDA has filed with the Library of Congress the annual reports for the following FDA advisory committees that held closed meetings during the period July 1, 1984, through June 30, 1985:

Center for Drugs and Biologics:
- Anesthetic and Life Support Drugs Advisory Committee,
- Anti-Infective Drugs Advisory Committee,
- Blood Product Advisory Committee
- Vaccines and Related Biological Products Advisory Committee.

Center For Devices and Radiological Health:
- Circulatory System Devices Panel,
- Immunology Devices Panel,
- Ophthalmic Devices Panel.

Annual reports are available for public inspection at (1) the Library of Congress, Newspaper and Current Periodical Reading Room, Rm. 1026, Thomas Jefferson Bldg., 2d and Independence Ave. SE., Washington, DC, (2) the Department of Health and Human Services Library, Rm. 1436, 330 Independence Avenue SW., Washington, DC, on weekdays between 9 a.m. and 4:30 p.m., and (3) the Dockets Management Branch (HFA-305), Food and Drug Administration, Rm. 4-62, 5600 Fishers Lane, Rockville, MD 20857, between 9 a.m. and 4:30 p.m., Monday through Friday.

Dated: August 14, 1985.

Mervin H. Shumate,

Acting Associate Commissioner for Regulatory Affairs.

[FR Doc. 85-19937 Filed 8-20-85; 8:45 am]

BILLING CODE 4160-01-M

[Docket No. 84D-0263]

Meningococcal Polysaccharide Vaccines; Availability of Guideline

AGENCY: Food and Drug Administration.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is announcing the availability of a guideline for the manufacture and release of Meningococcal Polysaccharide Vaccine and the bulk powders of each polysaccharide used for the manufacture of the vaccines.

ADDRESS: Written requests for a copy of the guideline or comments concerning the guideline to the Dockets Management Branch (HFA-305), Food and Drug Administration, Rm. 4-62, 5600 Fishers Lane, Rockville, MD 20857.

FOR FURTHER INFORMATION CONTRACT: Carl E. Frasch, Center for Drugs and Biologics (HFN-858), Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20205, 301-496-1920.

SUPPLEMENTARY INFORMATION: Meningococcal Polysaccharide Vaccine are biological products licensed under section 351 of the Public Health Service Act (42 U.S.C. 262).

FDA issued the first license for Meningococcal Polysaccharide Vaccine in 1974. FDA regulates production of these vaccines not only through standards in the license applications but also through requirements in the regulations on establishment and product licensing in 21 CFR Parts 600 and 601, the general biological standards in 21 CFR Part 610, the labeling requirements in 21 CFR Parts 201 and 202, and the current good manufacturing practice (CGMP) regulations in 21 CFR Part 211. FDA has not issued specific regulations for Meningococcal Polysaccharide Vaccines.

The Office of Biological Research and Review in FDA's Center for Drugs and Biologics has developed a guideline concerning the manufacture and release of Meningococcal Polysaccharide Vaccines and the polysaccharide bulk powders from which the individual polysaccharides used for making the

Exhibit 7

vaccines are derived. FDA is making the guideline available to provide guidance to the two currently licensed manufacturers and any possible new manufacturers concerning the production of safe and effective vaccines.

FDA is making the guideline available under 21 CFR 10.90(b). That regulation provides for FDA's use of guidelines to outline procedures or standards of general applicability that are acceptable to FDA for a subject matter that falls within the laws administered by FDA. Although guidelines are not legal requirements, a person may be assured that in following an agency guideline the procedures followed and standards used will be acceptable to FDA. A person may also choose to use alternative procedures or standards for which there is scientific rationale even though they are not provided for in a guideline. A person who chooses to use procedures or standards different from procedures or standards in a guideline may discuss the matter further with the agency to prevent an expenditure of resources for work that FDA may later determine to be unacceptable.

Interested persons may submit written comments on the guideline to the Dockets Management Branch (address above). Two copies of any comments are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. FDA will consider the comments when determining whether amendments or revisions to the guideline are warranted. The comments will assist the agency in developing additional standards for Meningococcal Polysaccharide Vaccines. Received comments may be seen in the office above between 9 a.m. and 4 p.m., Monday through Friday.

Dated: August 14, 1985.

Mervin H. Shumate,

Acting Associate Commissioner for Regulatory Affairs.

[FR Doc. 85-19936 Filed 8-20-85; 8:45 am]

BILLING CODE 4160-01-M

Public Health Service

Privacy Act of 1974; System of Records

AGENCY: Department of Health and Human Services; Public Health Service.

ACTION: Notification of a new system of records: 09-25-0156: "Records of Participants in Programs and Respondents in Surveys used to Evaluate Programs of the National Institutes of Health, HHS/NIH/OD."

SUMMARY: In accordance with the requirements of the Privacy Act, the Public Health Service (PHS) is published notice of a proposal to establish a new system of records, 09-25-0156, "Records of Participants in Programs and Respondents in Surveys Used to Evaluate Programs of the National Institutes of Health, HHS/NIH/OD." We are also proposing routine uses for this system.

The National Institutes of Health (NIH) will use the proposed system of records to support evaluation of the methods, materials, activities and services used by NIH in fulfilling its legislated mandate to conduct and support biomedical research into the causes, prevention and cure of diseases, to support training of research investigators, and to support communication of biomedical information.

PHS invites interested persons to submit comments on the proposed routine uses on or before September 20, 1985.

DATES: PHS has sent a Report of New System to the Congress and to the Office of Management and Budget (OMB) on August 12, 1985. The system of records will be effective 60 days from the date submitted to OMB unless PHS receives comments on the routine uses which would result in a contrary determination.

ADDRESS: Comments should be addressed to the NIH Privacy Act Coordinator at the address listed below. Comments received will be available for inspection weekdays between 9 a.m. and 3 p.m. in Room 3B11, Building 31, at that address.

FOR FURTHER INFORMATION CONTACT: Dr. Kenneth Thibodeau, NIH Privacy Act Coordinator, Building 31, Room 3B07, 9000 Rockville Pike, Bethesda, MD 20205, or call 301-496-2832. (This is not a toll free number.)

SUPPLEMENTARY INFORMATION: NIH was established to improve the health of the American people by conducting biomedical research, by funding such research through grants, contracts and other awards, and by promoting communication and dissemination of biomedical knowledge. NIH conducts reviews to determine how well these programs and projects achieve their goals.

The proposed system of records will cover cases where (1) NIH collects opinions, suggestions or other personal information concerning programs which do not use any system of records, or (2) program evaluation requires several categories of information which are not contained in any existing system.

Evaluation studies may take the form of surveys in which individuals who have or could participate in, contribute to, or benefit from NIH programs, provide opinions, suggestions or other information useful in evaluating the programs. For example, surveys of how much the public knows about certain methods for reducing the risk of disease may be used to evaluate dissemination of information about disease prevention. Other studies may use information from existing sources. For example, to evaluate research training programs, NIH may assemble information about publications by individuals who have received research training support, or from NIH grants files about research grants subsequently awarded to such individuals.

Evaluation studies may be conducted either by NIH staff or by other organizations under contract. Such contracts might be awarded when NIH staff is not available, when other organizations have better access to targeted populations, or when they can obtain or provide the desired information at lower cost or with less instrusion upon individuals.

The routine uses proposed for these systems are compatible with the purpose of evaluating NIH programs. The first two proposed routine uses are needed to accomplish the purpose of the system; that is, information would be disclosed under these routine uses to conduct, review, analyze or follow-up evaluation studies. The third proposed routine use for disclosure to a congressional office acting on behalf of an individual would not violate the privacy of an individual because such disclosure would be made only pursuant to a request initiated by the individual.

The proposed system will cover evaluation studies throughout NIH. Records in this system will be organized and maintained according to the particular evaluation study of which they are a part. NIH will not enter records into a general or comprehensive data base, nor create any overall index to the separate sets of records used in different evaluation projects; however, NIH is treating the separate sets of records as a single system under the Privacy Act (1) because all of the sets of records serve the same purpose, (2) in order to apply consistent policies and practices in the maintenance of such records, and (3) to make it easier for subject individuals to obtain notification of or access to their records.

When it becomes effective, the proposed system will subsume records currently covered by three existing systems: 09-25-0147, "Records of

GUIDELINES FOR THE PRODUCTION OF

MENINGOCOCCAL POLYSACCHARIDE VACCINES

Docket No. 84D-0263

Notice of Availability Published August 21, 1985
FR 50 (162) 33852

Center for Drugs and Biologics
Office of Biologics Research and Review
Food and Drug Administration
5600 Fishers Lane
Rockville, Maryland 20857

Guidelines for meningococcal vaccine,
groups A, C. Y, and W135 polysaccharides combined

A. Testing procedures

1. Group A, C, Y, and W135 final bulk powders used for formulation of the final container should pass all tests described in section A.1. of the release specifications for the individual polysaccharides.

2. The following tests should be conducted using material in final containers:

a. Quantitation: The final container should contain 50.0 µg ±20.0% per dose of groups A, C, Y, and W135 polysaccharides as determined by the rocket immunoelectrophoretic technique of the Office of Biologics Research and Review. Other quantitative immunochemical procedures, shown to yield results equivalent to the above technique, would be acceptable after being submitted in writing and approved by the Director, Office of Biologics Research and Review. The reference standard for each meningococcal group polysaccharide to be used for this quantitative procedure should pass all tests required of the final bulk powders (see A.1. above).

b. Stabilizer content: Lactose should be present at a concentration of 2.5-5.0 mg per human dose of the vaccine.

c. Pyrogenic substances: The vaccine should meet the standards of 21 CFR 610.13(b)(3) in a test dose of 0.10 micrograms vaccine (dry weight) per milliliter per kilogram of rabbit body weight.

d. Molecular sizing: Molecular sizing of meningococcal groups A, C, Y, and W135 polysaccharides should be determined by Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ) gel filtration, using the procedure of the Office of Biologics Research and Review or a procedure shown to yield equivalent results. The individual polysaccharides should be quantitated by an immunoelectrophoretic or equivalent procedure. The recommended specifications for molecular size are maximum Kd values on CL-2B as follows: Group A, 0.70, group C, 0.70, group Y, 0.57, and group W135, 0.68.

e. Moisture: The moisture content of the vaccine in final containers should be determined by the gravimetric procedure (21 CFR 610.13) or an equivalent procedure. The test should be performed on one vial per thousand up to a maximum of 10 vials but not less than 5 vials taken at random throughout the filling lot. The average residual moisture should not exceed 1.0%.

f. General safety: A general safety test must be conducted as provided in 21 CFR 610.11.

g. Sterility: Requirements with respect to sterility are set forth in 21 CFR 610.12.

h. Identity: Requirements with respect to establishment of identity are set forth in 21 CFR 610.14.

i. Clinical potency: A clinical potency test should normally be conducted on at least two of the first three first-filling lots of A, C, Y, W135 tetravalent vaccine.

Samples of sera from each subject taken immediately prior to injection and again 3 to 4 weeks after immunization should be assayed for bactericidal antibodies.

The bactericidal assay should be performed with paired sera from each subject in serial two-fold dilutions against the following meningococcal strains: for group A strain A1, for group C strain C-11, for group Y strain S-1975, and for group W135 strain S-4383. The antibody titer should be expressed as the reciprocal of the highest dilution that effects 50% or greater killing of the test organisms. At least 90% of the subjects should show a four-fold or greater rise to all four strains after immunization. If the sera from less than 90% but greater than 80% of the subjects show such a rise, one retest of the product using not less than 25 human subjects may be performed. The sera from at least 90% of all subjects in the two tests combined should show a four-fold or greater antibody increase.

At least 2 milliliters each of pre-and post-immunization sera from each of the human subjects tested should be saved by the manufacturer. The Office of Biologics Research and Review may request these samples to be submitted for testing. These sera will contain no additives, and should be stored and shipped in a manner which prevents the growth of possible microbial contaminants.

B. Manufacturing protocol. For each lot of vaccine, a release protocol which consists of a summary of the history of manufacture, filling date, date of performance and result(s) of each required test and the calculation of the actual fill weight of each of polysaccharide Group A, Group C, Group Y and Group W135 antigens should be submitted to the Director, Office of Biologics Research and Review. Protocols are to include all clinical potency results and any adverse reactions observed in the clinical test (A.2.g.), if performed. Protocols for each lot of combined A, C, Y, W135 vaccine should include the lot number of the final dry bulk for each of the polysaccharides used in formulation of the vaccine lot.

C. Samples. For each lot of vaccine, a sample of no less than 60 milligrams of the vaccine distributed in no fewer than 12 final containers, and an appropriate amount of diluent should be submitted to the Director, Office of Biologics Research and Review. Additional samples include (also described in final bulk release specifications):

(i) 75 milligrams of powder from the Group A, Group C, Group Y, and Group W135 final dry bulks for the first lot of vaccine derived from these bulks.

(ii) 10 milligrams of powder for each subsequent vaccine lot utilizing the final bulk powder.

D. Dating period

1. The in-house storage period and storage temperature of groups A, C, Y and W135 combined vaccine in filled but undated final containers should be no longer than thirty-six (36) months at -20°C or colder from date of filling into final containers.

2. The dating period and storage temperature of labeled and packaged final container vaccine should be twenty-four (24) months at 2-8°C from the date of initiation by the manufacturer of the last valid potency tests (A.2. a through g), and the term of the dating period should not exceed sixty (60) months from date of filling.

INFECTION AND IMMUNITY, Nov. 1999, p. 5664–5675 Vol. 67, No. 11
0019-9567/99/$04.00+0

Exhibit 8

Differences in Surface Expression of NspA among *Neisseria meningitidis* Group B Strains

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Received 7 June 1999/Returned for modification 13 July 1999/Accepted 6 August 1999

NspA is a highly conserved membrane protein that is reported to elicit protective antibody responses against *Neisseria meningitidis* serogroups A, B and C in mice (D. Martin, N. Cadieux, J. Hanel, and B. R. Brodeur, J. Exp. Med. 185:1173–1183, 1997). To investigate the vaccine potential of NspA, we produced mouse anti-recombinant NspA (rNspA) antisera, which were used to evaluate the accessibility of NspA epitopes on the surface of different serogroup B strains by an immunofluorescence flow cytometric assay and by susceptibility to antibody-dependent, complement-mediated bacteriolysis. Among 17 genetically diverse strains tested, 11 (65%) were positive for NspA cell surface epitopes and 6 (35%) were negative. All six negative strains also were resistant to bactericidal activity induced by the anti-rNspA antiserum. In contrast, of the 11 NspA surface-positive strains, 8 (73%; $P < 0.05$) were killed by the antiserum and complement. In infant rats challenged with one of these eight strains, the anti-rNspA antiserum conferred protection against bacteremia, whereas the antiserum failed to protect rats challenged by one of the six NspA cell surface-negative strains. Neither NspA expression nor protein sequence accounted for differences in NspA surface accessibility, since all six negative strains expressed NspA in outer membrane preparations and since their predicted NspA amino acid sequences were 99 to 100% identical to those of three representative positive strains. However, the six NspA cell surface-negative strains produced, on average, larger amounts of group B polysaccharide than did the 11 positive strains (reciprocal geometric mean titers, 676 and 224, respectively; $P < 0.05$), which suggests that the capsule may limit the accessibility of NspA surface epitopes. Given these strain differences in NspA surface accessibility, an rNspA-based meningococcal B vaccine may have to be supplemented by additional antigens.

With the control of *Haemophilus influenzae* type b disease by vaccination, *Neisseria meningitidis* has emerged as the most common cause of bacterial meningitis in children and young adults (45, 56). Strains of *N. meningitidis* can be divided into 12 serogroups based on chemically and antigenically distinctive polysaccharide capsules (14). Five serogroups, designated A, B, C, Y, and W-135, account for virtually all disease-producing isolates (67).

Plain meningococcal polysaccharide vaccines are currently available for the prevention of disease caused by serogroups A, C, Y, and W-135 (18). These vaccines are efficacious in older children and adults but not in infants, the age group at greatest risk of acquiring the disease (18). Second-generation polysaccharide-protein conjugate vaccines are in various stages of clinical development. These vaccines are much more immunogenic in infants than are plain polysaccharide vaccines (16, 28, 32). It is likely, therefore, that highly effective meningococcal conjugate vaccines for the prevention of disease caused by serogroups A, C, Y, and W-135 strains will be licensed for routine use in children in the near future. However, attempts to develop a vaccine for the prevention of group B meningococcal disease have been problematic. Group B strains are a common cause of disease, currently accounting for approximately one-third of disease-producing isolates in the United States (10, 11), half in the United Kingdom (49), and up to 80 to 90% in The Netherlands (55). Therefore, lack of an effective vaccine for the prevention of serogroup B disease will substantially limit the overall effectiveness of a vaccination program for control of meningococcal disease.

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To date, experimental meningococcal B vaccines have been designed to elicit serum antibody responses either to the group B capsule or to noncapsular antigens. Capsule-based vaccines are limited by poor immunogenicity, even when the polysaccharide is conjugated to a protein carrier (15, 26). The group B polysaccharide consists of $\alpha(2\rightarrow8)$*N*-acetylneuraminic acid (polysialic acid). The poor immunogenicity of this polysaccharide is attributed to immunologic tolerance as a result of exposure to cross-reacting polysialic acid expressed by a variety of host tissues, particularly the neural cell adhesion molecule (17, 23). In an innovative strategy for overcoming immunologic tolerance to the meningococcal B capsule, Jennings et al. substituted *N*-propionyl (*N*-Pr) groups for *N*-acetyl groups and conjugated the *N*-Pr meningococcal B polysaccharide derivative to a carrier protein (25). The resulting conjugate vaccine was highly immunogenic in experimental animals, eliciting immunoglobulin G (IgG) antibodies that activated complement-mediated bacteriolysis in vitro and passively protected experimental animals infected with meningococcal B bacteria. However, a subset of the antibodies elicited by this vaccine were also autoantibodies (20, 21). Given the unknown risks of a vaccine that elicits anticapsular antibodies that are also autoantibodies, recent efforts to develop a vaccine for prevention of serogroup B disease have focused on the use of alternative noncapsular antigens as vaccine components.

Non-capsular-antigen-based vaccines include outer membrane vesicles (5, 7, 42, 59, 68), specific outer membrane proteins such as PorA (6, 12, 24, 40, 41, 51, 62, 63), iron-regulated proteins such as transferrin binding protein (3, 31, 43), outer membrane opacity proteins such as Opa and Opc (39, 50), and detoxified lipopolysaccharide (64).

While vaccines based on outer membrane vesicles or recombinant PorA appear to be safe and can induce protective antibody in humans (18, 59), the bactericidal antibody responses

TABLE 1. Reactivity of anti-rNspA polyclonal antiserum with NspA exposed on the surface of live, encapsulated, *N. meningitidis* serogroup B bacteria in relation to susceptibility to bacteriolysis and capsule production

N. meningitidis serogroup B[a]					NspA cell surface reactivity[d]	Anti-rNspA bactericidal activity[e] (reciprocal titer)	Polysaccharide capsule production (reciprocal/titer)[f]
Strain	Country	Yr	Serologic classification[c]	ET complex			
SWZ107[b]	Switzerland	1980	4:P1.2	104	Positive	≥64	28 ± 4
NG6/88[b]	Norway	1988	NT: P1.1	173	Positive	4	115 ± 23
CU385	Cuba	1980	4: P1.15	5	Positive	4	116 ± 1
IH5341	Finland	1985	15:P1.7,16	ND	Positive	16	176 ± 61
BZ198	The Netherlands	1986	NT: P−	154	Positive	≥64	362 ± 1
NMB	United States	1968	2b:P1.2,5	ND	Positive	16	244 ± 20
8047	United States	1978	2b: P1.2	ND	Positive	16	1,125 ± 50
H44/76	Norway	1976	15: P1.7, 16	5	Positive	24	99 ± 16
1000[b]	USSR	1989	NT:P1.5	61	Positive	<4	287 ± 12
S3446	United States	1972	14: P1.23, 14	11 (A1 cluster)	Positive	<4 (static=16)	585 ± 151
H355	Norway	1973	15: P1.15	11 cluster	Positive	<4 (static=4)	656 ± 141
BZ232[b]	The Netherlands	1964	NT:P1.2	76	Negative	<4	1,493 ± 18
NG3/88[b]	Norway	1988	8: P1.1	A4 cluster	Negative	<4	498 ± 105
MC58	United Kingdom	1985	15:P1.7,16b	5	Negative	<4	627 ± 121
M136	United States	1968	11: P1.15	D1 cluster	Negative	<4	1,056 ± 81
M986	United States	1963	2a: P1.5, 2	B2 cluster	Negative	<4	1,442 ± 206
NGP165	Norway	1974	NT:P1.2	37	Negative	<4	138 ± 6

[a] Strains were obtained from the collection of C. Frasch, Center for Biologics Evaluation and Research, Rockville, Md.; 8047 was obtained from W. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.; MC58 is the strain selected by The Institute of Genomic Research for genomic sequencing; IH5341 was obtained from M. Sarvas, National Public Health Institute, Helsinki, Finland; and the remaining strains are from the collection described by Seiler et al. (57). ET data are from Caugnant et al. (9) and Seiler et al. (57).

[b] Strain has been characterized further by multilocus sequence typing (33).

[c] See reference 19 for a definition of the meningococcal serotype and subtype classification system.

[d] By indirect-fluorescence flow cytometry with anti-rNspA antisera.

[e] Dilution of anti-rNspA antisera that when incubated for 60 min with bacterial cells and 20% human complement yielded a 50% decrease in CFU per milliliter compared to that at time zero. "Static" refers to strains that were inhibited but not killed in the assay (≥50% but <100% survival at 60 min.).

[f] Titer defined as dilution of capsule extract giving 50% inhibition of antibody binding to meningococcal B polysaccharide antigen in an ELISA (see Materials and Methods). Results are means and standard errors.

are strain specific, especially in infants (18, 59). Therefore, a multivalent PorA approach is required. In initial clinical studies of one multivalent vaccine, an outer membrane vesicle vaccine containing multiple PorA recombinant proteins, only modest serum bactericidal titers were elicited in infants, and there appeared to be antigenic interference between some of the serotype antigens (52). Given the propensity for meningococcal disease during nonepidemic periods to be caused by multiple strains or strain variants (53), together with the frequent temporal shifts in the predominant strains in a community, it will be difficult to develop a universal meningococcal B vaccine with only outer membrane protein components that are highly variable from strain to strain.

Recently, Martin et al. described a membrane protein, designated neisserial surface protein A (NspA), which was reported to be highly conserved across *Neisseria* strains (36) and elicited serum bactericidal antibody responses in mice to *N. meningitidis* strains from serogroups A, B, and C (36). Immunized mice also were reported to be protected from a lethal challenge with live meningococcal B organisms (36). Thus, NspA appears to represent a novel and promising vaccine candidate.

To investigate further the vaccine potential of NspA, we cloned the *nspA* gene, expressed NspA in *Escherichia coli*, and produced mouse polyclonal anti-recombinant NspA (anti-rNspA) antisera. The resulting antisera were used to evaluate the accessibility of NspA epitopes on the surface of different strains of live encapsulated *N. meningitidis* group B bacteria and the susceptibility of these strains to antibody-dependent, complement-mediated bacteriolysis. In this report, we confirm that the NspA gene and protein sequences are highly conserved among genetically divergent strains of *N. meningitidis* group B. However, despite conservation of the protein sequence and expression, we found strain differences in the surface accessibility of NspA epitopes and in the susceptibility of different strains to anti-NspA bactericidal activity.

MATERIALS AND METHODS

Bacterial strains. The *N. meningitidis* strains studied were isolated from patients residing in different countries over a period of more than 30 years (Table 1). The specific strains were selected to be representative of widely divergent "clonal" groups, as defined by multilocus isoenzyme typing and/or multilocus sequence typing (33, 57). Strain M7, which is derived from strain NMB, contains a transposon insertion that blocks capsular polysaccharide biosynthesis (58). All of the other strains were encapsulated.

Cloning, expression, and purification of rNspA and HisTag-NspA. Based on the nucleotide sequence published by Martin et al. (36), the following primers that are complementary to opposing strands at the 5′ and 3′ ends of the *nspA* gene, respectively, were synthesized: 5′-ACAGCAGGATCCTTTAACGGATTC-3′ and 5′-GTGGATGAAGCTTTGGACATTTC-3′. In addition, the primers contained base substitutions that created cleavage sites for *Bam*HI and *Hin*dIII at the 5′ and 3′ ends, respectively, of the *nspA* gene. By using the method described for DNA sequencing below, these primers were used in PCR to amplify a 743-bp DNA segment from the genome of *N. meningitidis* 8047. The fragment, which includes the wild-type promoter region, was subsequently cloned into the multicopy plasmid pSK(+) (Stratagene, San Diego, Calif.). In addition, we obtained as a gift from R. Rappuoli (Chiron Vaccines, Siena, Italy) a plasmid that contains a truncated version of the *nspA* gene (pTrc.NspA.1). In this construct, the portion of the gene encoding the signal sequence has been replaced with DNA encoding a series of six histidine residues (expression vector pTrcHis [Invitrogen, Carlsbad, Calif.]).

Recombinant HisTag-NspA was expressed in *Escherichia coli* BL21(DE3) (Stratagene) transformed with plasmid pTrc.NspA.1 by induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). HisTag-NspA protein was purified by Ni-nitrilotriacetic acid Sepharose chromatography of cell lysates solubilized in 6 M guanidine hydrochloride by using the materials and methods provided in the QiaExpress kit (Qiagen, Valencia, Calif.). Recombinant wild-type NspA, expressed under the control of its own promoter from plasmid pGMS 1.0, was also produced in *E. coli* BL21(DE3). The protein was excreted into the culture medium and was partially purified by precipitation of culture medium with ammonium sulfate (55%, wt/vol).

DNA preparation and sequencing. Multiple and single clones of each strain were grown to an absorbance at 620 nm (A_{620}) of 1 in 7 ml of Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with 0.2% glucose. Genomic DNA was prepared with a commercial kit (Qiagen) as specified by the manufacturer for the preparation of genomic DNA from bacteria (47). DNA used for sequencing was obtained by PCR amplification of *nspA* in the genomic DNA preparation with the primers described above. The *Taq* polymerase and other reagents used for PCR were from Qiagen. The protocol consisted of a denaturation step (94°C for 5 min) followed by 35 cycles of amplification (94°C for 1 min, 60°C for 2 min, and 72°C for 1.5 min) and 1 cycle of extension (72°C for 5 min). Amplified DNA was purified with a QIAquick PCR purification kit (Qiagen) as specified by the manufacturer (48). DNA sequencing of each clone was performed by McConnel Research (San Diego, Calif.) with the primers given above. For determination of the sequence of the *nspA* gene of each strain, two or three independent PCR products from single clones were sequenced on both strands. Sequence alignments and translations were performed with MacVector software (Oxford Molecular, Cambridge, Mass.).

Polyclonal anti-rNspA antisera. To prepare antisera against NspA, 50 μg of purified HisTag-NspA or partially purified wild-type rNspA expressed from its own promoter was used to immunize groups of 4- to 6-week old female CD-1 mice (five to eight mice per group). Injections were given intraperitoneally (i.p.). Complete Freund's adjuvant was used for the first dose, and incomplete Freund's adjuvant was used for two subsequent booster doses given at 2- to 3-week intervals. As controls, groups of mice were immunized with the same adjuvants combined with proteins precipitated from culture medium supernatant of *E. coli* BL21(DE3) cells transformed with the parent plasmid pSK(+), which lacks the *nspA* gene. In a few experiments, we also immunized mice intramuscularly with 50 μg of rNspA given with 5 μg of purified QuilA (gift of G. Ott, Chiron Corp., Emeryville, Calif.) as the adjuvant, and we also compared the responses of female BALB/c and CD-1 mice. For assessment of serum antibody responses, individual mouse sera obtained 2 to 4 weeks after the third immunization were pooled.

Binding of antisera to the surface of live encapsulated meningococci. The ability of antisera elicited by the recombinant NspA constructs to bind to the surface of pathogenic strains of *N. meningitidis* group B was determined by flow cytometric detection of indirect fluorescence assay, performed as described previously (21). In brief, bacterial cells were grown to mid-log phase in Mueller-Hinton broth, harvested by centrifugation, and resuspended in blocking buffer (phosphate-buffered saline [PBS] containing 1% [wt/vol] bovine serum albumin and 0.2% [wt/vol] sodium azide) at a density of ~10^8 cells per ml. Dilutions of test or control antiserum (typically 1:10 to 1:100) were then added and allowed to bind to the cells, which were maintained on ice for 2 h. Following two washes with blocking buffer, the cells were incubated with the fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (heavy plus light chains) (Jackson ImmunoResearch, West Grove, Pa.) and fixed with 0.25% formaldehyde in PBS buffer, and the bacterial cells were analyzed by flow cytometry.

Positive control antibodies included meningococcus-specific serotyping or subtyping monoclonal antibodies (MN2C3B and MN16C13F4 [Rijksinstituut Voor Volksgezondheid en Mileu, Bilthoven, The Netherlands]) and SEAM 12, an anti-polysaccharide monoclonal antibody (MAb) that is specific for encapsulated group B strains (21). The negative control consisted of a mouse IgG MAb (VIG10) of irrelevant specificity.

Complement-dependent bactericidal antibody activity. The bactericidal assay was adapted from the method previously described from this laboratory (35) with the following modifications. After overnight growth on chocolate agar, several colonies were inoculated into in Mueller-Hinton broth (starting A_{620} of ~0.1) and the test organism was grown for approximately 2 h to an A_{620} of ~0.6. After the bacteria were washed twice in Gey's buffer, approximately 300 to 400 CFU was added to the reaction mixture. The final reaction mixture of 60 μl contained 20% (vol/vol) complement and serial twofold dilutions of test sera or control MAbs in Gey's buffer (instead of barbital buffer as previously described by Mandrell et al. [35]). The complement source was human serum from a healthy adult with no detectable anticapsular antibody to group B polysaccharide when tested by the enzyme-linked immunosorbent assay (ELISA) (22) and no detectable intrinsic bactericidal activity at a final concentration of 20 or 40% against the test strain. In preliminary experiments with a panel of test sera, this complement source gave comparable bactericidal titers to those obtained with agammaglobulinemic serum as the complement source. Serum bactericidal titers were defined as the serum dilution (or antibody concentration) resulting in a 50% decrease in CFU per milliliter after a 60-min incubation of bacteria in the reaction mixture, compared to the control CFU per milliliter at time zero. Typically, bacteria incubated with the negative control antibody and complement showed a 150 to 200% increase in CFU per milliliter during the 60 min of incubation.

Animal protection. The ability of the anti-rNspA antiserum to confer passive protection against *N. meningitidis* group B bacteremia was tested in infant rats challenged i.p. by a method adapted from that of Saukkonen et al. (54). In brief, 6- to 7-day-old pups from six litters of outbred Wistar rats (Charles River, Hollister, Calif.) were randomly redistributed to the nursing mothers. Groups of five or six animals were challenged i.p. with 100 μl of approximately 5×10^3 CFU of *N. meningitidis* group B bacteria. Two strains, M986 and 8047, each of which had been passaged three times in infant rats, were tested. The bacteria isolated from blood cultures after the third pass were grown on chocolate agar overnight and stored frozen at −70°C in vials containing sterile skim milk. On the day of the experiment, the bacteria were grown, washed, and resuspended in Gey's buffer, as described above for the bactericidal assay. Immediately before administration to the animals, the bacterial suspension was mixed with different dilutions of test or control antiserum or MAb. At 18 h after the bacterial challenge, blood specimens were obtained by puncturing the heart with a syringe and needle containing approximately 25 U of heparin without preservative (Fujisawa USA, Deerfield, Ill.). Aliquots of 1, 10, and 100 μl of blood were plated onto chocolate agar. The CFU per milliliter of blood was determined after overnight incubation of the plates at 37°C in 5% CO_2.

Membrane preparations. Single colonies were grown to an A_{620} of 0.7 to 0.9 in 7 ml of Mueller-Hinton broth (Difco) supplemented with 0.2% glucose. The 7-ml culture was then used to innoculate 200 ml of the same medium prewarmed to 37°C. After growth of the culture to an A_{620} of 0.9, the bacteria were collected by centrifugation at 5,000 × *g* for 15 min. The cell pellets were frozen at −20°C until used for preparation of membrane proteins. Sodium lauroyl sarcosinate-insoluble extracts of bacterial membranes were prepared as described previously for *H. influenzae* type b (4). Briefly, the cell pellets were resuspended in 10 ml of HEPES buffer (pH 7.4) at 4°C. The cell suspension was then sonicated on ice with several 15-s bursts from a microprobe sonifier (Branson, Danbury, Conn.). Cell debris was removed by centrifugation at 5,000 × *g* for 20 min, and the membrane vesicles in the supernatant were obtained by ultracentrifugation at 100,000 × *g* for 1 h at 4°C. The resulting membrane pellet was resuspended in 2 ml of HEPES buffer (pH 7.4) containing 1% (wt/vol) sodium lauroyl sarcosinate (Sigma, St. Louis, Mo.) at ambient temperature. After a 30-min incubation, the sodium lauroyl sarconsinate-insoluble fraction was collected by ultracentrifugation at 100,000 × *g* for 2 h.

SDS-PAGE and Western blots. NspA protein preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21% polyacrylamide) as described by Laemmli (27) with a Mini-Protean II electrophoresis apparatus (Bio-Rad, Richmond, Calif.). Samples were suspended in SDS sample buffer (0.06 M Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10 μg of bromophenol blue per ml) and either heated to 100°C for 5 min or, in some experiments, left at ambient temperature for 5 min before being loaded directly onto the gel. For Western blots, the gel was equilibrated with buffer (48 mM Tris-HCl, 39 mM glycine [pH 9.0], 20% [vol/vol] methanol) and transferred to a nitrocellulose membrane (Bio-Rad) by using a Trans-Blot (Bio-Rad) semidry electrophoretic transfer cell. The nitrocellulose membranes were blocked with 2% (wt/vol) skim milk in PBS containing 0.2% (wt/vol) sodium azide. Anti-HisTag-NspA antisera were diluted in PBS containing 1% (wt/vol) bovine serum albumin, 1% (wt/vol) Tween 20, and 0.2% (wt/vol) sodium azide. Bound antibody was detected with rabbit anti-mouse IgG-, IgA-, and IgM-alkaline phosphatase conjugate polyclonal antibody (Zymed, South San Francisco, Calif.) and Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) substrate (Sigma).

Quantitation of capsular polysaccharide antigen. The amount of capsular polysaccharide produced by each strain was determined by an inhibition ELISA. First, extracts of capsular polysaccharide were prepared based on a method described by Corn et al. (13). In this method, individual bacterial clones were grown for 1.5 to 2 h in 7 ml of Mueller-Hinton broth to an A_{620} of 0.5 to 0.7. The bacteria were collected by centrifugation at 5,000 × *g* for 15 min. The cells were washed once in 0.6 ml of 10 mM HEPES (pH 8.0) and then resuspended in 0.6 ml of the same buffer containing 10 mM EDTA and incubated at 37°C for 1 h. The cells were pelleted at 10,000 × *g* for 1 min, and the relative amount of meningococcal B polysaccharide antigen released into the supernatant was determined by an inhibition ELISA, performed as described by Azmi et al. (2). The solid-phase antigen in the ELISA was meningococcal B polysaccharide-adipic-dihydrazide-biotin absorbed to avidin-coated microtiter plates as previously described (22). The meningococcal B polysaccharide-reactive human paraprotein LIP (2), at concentration of 0.2 μg/ml, was used as the primary antibody. In the absence of inhibitor, this concentration of antibody was sufficient to give an A_{405} of ~0.7 to 1.0 after a 30-min incubation with substrate (2). The titer of polysaccharide released into the supernatant was measured by determining the dilution of supernatant that resulted in 50% inhibition of antibody binding. Controls in this assay included an EDTA extract prepared from strain M7, which does not produce any capsular polysaccharide (58), and purified meningococcal B polysaccharide. To ensure that all of the capsular polysaccharide was released by the EDTA treatment, the same inhibition ELISA was performed with the cell pellet resuspended in the same buffer and volume as the capsule extract. The observable inhibitory activity from the cell pellet was between 0 and 10% of the activity observed in the capsule extracts, with the latter, higher percentage coming from cell pellets of strains that produce the largest amounts of capsule.

RESULTS

Figure 1A shows a Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). The sample in lane 1 contains the HisTag-NspA, purified by Ni-nitrilotriacetic acid Sepharose chromatography of cell lysates solubilized in 6 M guanidine

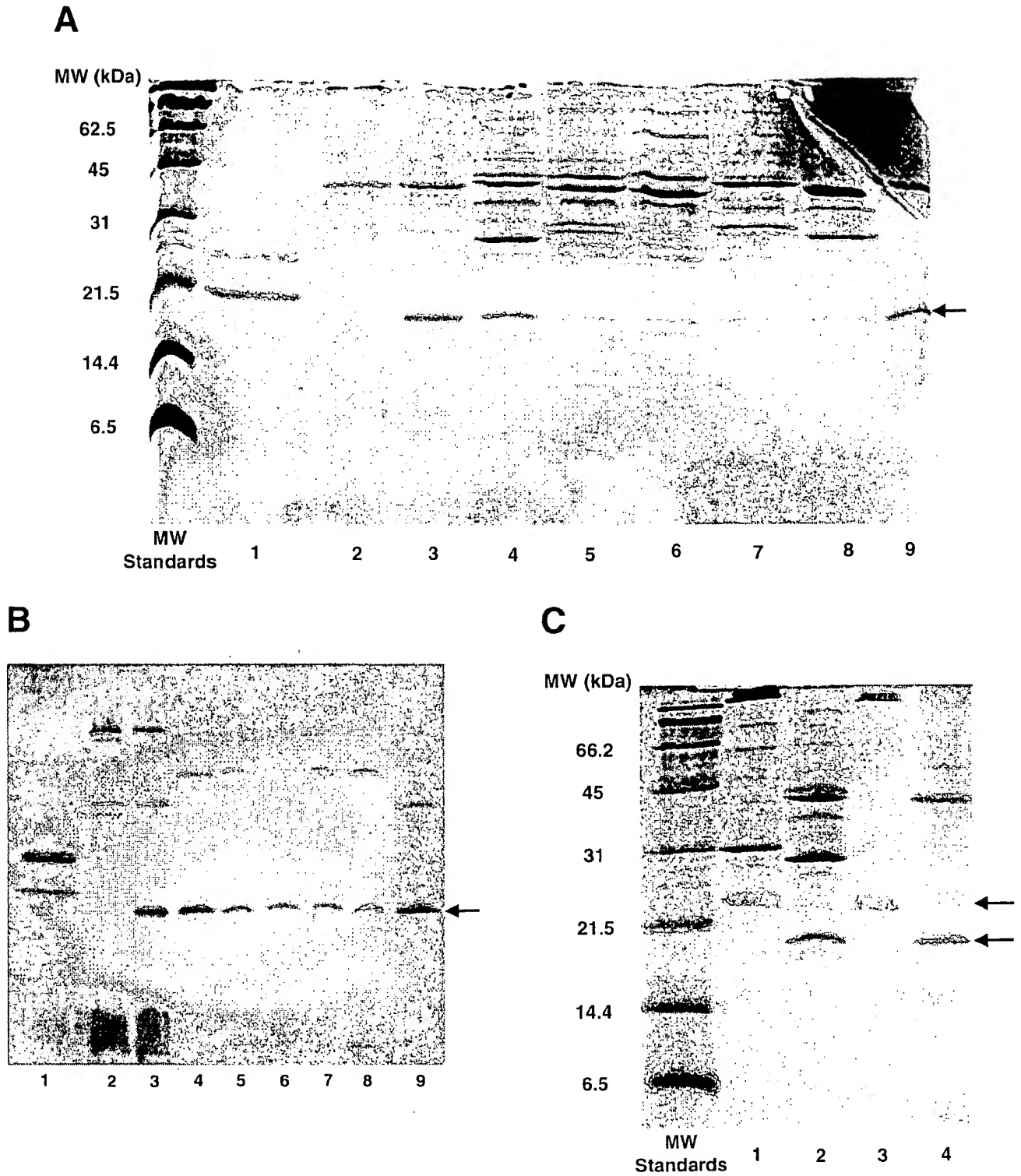

FIG. 1. (A) Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). Lanes: 1, HisTag-NspA eluted from the Ni-NTA Sepharose metal affinity column with 8 M urea–0.1 M NaH_2PO_4–0.01 M Tris-HCl buffer at pH 4.5; 2, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pSK(+) (the parent plasmid without the *nspA* gene); 3 and 9, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pGMS1.0 [the pSK(+) plasmid containing the wild-type *nspA* gene without the His tag]; production of rNspA is evident by the appearance of a protein with an apparent molecular mass of 18.6 kDa (arrow); 4 to 8, lauroyl sarcosinate-insoluble outer membrane preparations of meningococcal B strains 8047 (lane 4), CU385 (lane 5), NG6/88 (lane 6), M986 (lane 7), and M136 (lane 8). (B) Binding of anti-HisTag-NspA antiserum as shown by Western blotting. Lanes are identical to those in panel A. Except for HisTag-NspA, which appears as two immunoreactive bands at higher apparent molecular masses (see the text), the presence of NspA in each of the samples is indicated by the arrow at an apparent molecular mass of 18.6 kDa. (C) Coomassie blue-stained SDS-PAGE gel (21% polyacrylamide). Lanes: 1, lauroyl sarcosinate-insoluble membrane proteins from strain 8047 that has not been heated in sample buffer; 2, sample comparable to that in lane 1 that was heated to 100°C for 5 min; 3, ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pGMS 1.0 [the pSK(+) plasmid containing the wild-type *nspA* gene without the His tag] that has not been heated in sample buffer; 4, a sample comparable to that in lane 3 that was heated to 100°C for 5 min. The arrows indicate the shift from the higher apparent molecular weight to the expected molecular weight of NspA, as a result of heating the sample in sample buffer.

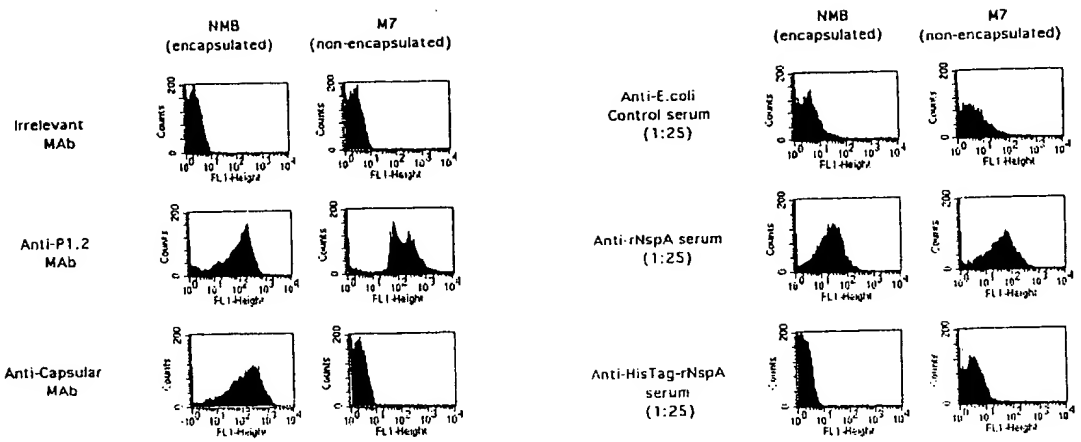

FIG. 2. Binding of polyclonal anti-rNspA antisera and control MAbs to encapsulated and nonencapsulated strains of live meningococcal B bacteria as determined by indirect-fluorescence flow cytometry. Strain M7 is a nonencapsulated mutant derived from strain NMB by transposon insertion (58). All antisera were tested at a dilution of 1:25. The control MAbs and antisera include VIG10, a murine MAb with an irrelevant specificity; *N. meningitidis* subtype MAb anti-P1.2 reagent (Rijksinstituut Voor Volksgezondheid en Mileu, Bilthoven, The Netherlands); and SEAM 3, an anti-capsule-specific murine MAb (21); as well as pooled sera from mice immunized with proteins from the supernatant culture of *E. coli* BL21(DE3), containing the vector that lacks the *nspA* gene (anti-*E. coli* control). The test antisera include anti-rNspA antisera elicited by immunization of mice with ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) that expresses rNspA from plasmid pGMS 1.0 and polyclonal anti-HisTag-NspA antiserum.

hydrochloride, which resolves as two bands with apparent molecular masses of 20.2 and 24.5 kDa. These bands appear to be different forms of HisTag-NspA rather than different proteins, since both the number and the apparent molecular masses of the bands in the HisTag-NspA sample are dependent on the content of SDS and EDTA in the sample buffer (data not shown).

According to Martin et al. (36), NspA is associated with the outer membrane. However, recombinant NspA produced in *E. coli* (without the His tag) is secreted into the culture medium. In their experiments, the "secreted" fraction of the rNspA protein was affinity purified and used as a vaccine. Figure 1A, lane 2, shows the proteins present in ammonium sulfate-precipitated culture medium from *E. coli* BL21(DE3) transformed with pSK(+), the parent cloning vector without the *nspA* gene. Lanes 3 and 9 contain duplicate samples of the ammonium sulfate-precipitated culture medium from the same *E. coli* strain transformed with plasmid pGMS1.0, which contains the wild-type *nspA* gene. The presence of rNspA is evident by the appearance of a protein with an apparent molecular mass of 18.6 kDa, corresponding to the molecular mass of NspA predicted from the amino acid sequence. This protein is not present in the control (lane 2). Lanes 4 to 8 contain sodium lauroyl sarcosinate-insoluble membrane proteins from strains 8047, CU385, NG6/88, M986, and M136, respectively. All contain a protein having the same mobility (18.6 kDa) as rNspA (lanes 3 and 9).

Confirmation that the 18.6-kDa protein observed in Fig. 1A, lanes 3 to 9, is NspA was obtained by Western blot analysis with anti-HisTag-NspA polyclonal serum as the detecting antibody. The results are shown in Fig. 1B. The 18.6-kDa bands present in Fig. 1B, lanes 3 to 9, are reactive with the anti-HisTag-NspA sera. Note that in lanes 2 and 3 the anti-HisTag NspA antisera also react with higher-molecular-weight proteins. However, these cross-reacting higher-molecular-weight proteins were present in both the control (i.e., without NspA) and rNspA-containing preparations.

When analyzed by SDS-PAGE, NspA is reported to have two forms, a 22-kDa diffuse band and a 18-kDa band (36). However, we found that these two forms resulted because NspA is a heat-modifiable protein. Figure 1C shows an SDS-PAGE gel (21% polyacrylamide) of sodium lauroyl sarcosinate-insoluble outer membrane proteins from strain 8047 and partially purified rNspA. Lane 3 contains rNspA that has not been heated in sample buffer. A diffuse band with an apparent molecular mass of 23 kDa is present. Although not shown, a comparable band was not present in proteins present in ammonium sulfate-precipitated culture medium from *E. coli* containing the parent cloning vector without the *nspA* gene. As shown in Fig. 1C, lane 4, when the partially purified rNspA was heated to 100°C in sample buffer, the 23-kDa band disappeared and was replaced by a protein with an apparent molecular mass of 18.6 kDa. A similar shift in mobility was observed in the respective proteins present in the detergent-insoluble outer membrane protein preparation from strain 8047 (compare lanes 1 and 2). In both the rNspA preparation and the outer membrane preparation from strain 8047, the 18.6-kDa band was recognized by the anti-HisTag-NspA antiserum in the Western blot (Fig. 1B). The anti-HisTag NspA antiserum also recognized the respective 23-kDa bands in samples that had not been heated (data not shown).

Binding of anti-rNspA serum to native NspA on the surface of live meningococcal B bacteria. Binding of serum antibodies to the cell surface of live meningococci was measured by flow cytometric detection of indirect immunofluorescence (21). Figure 2 shows the results of a typical experiment examining antibody binding to two test strains: NMB, a fully encapsulated *N. meningitidis* group B strain, and M7, a transposon-containing capsule-deficient mutant of NMB (58). As expected, the anti-group B polysaccharide MAb (SEAM 3) (21) binds only to the encapsulated strain whereas as the positive control anti-P1.2 (PorA) MAb binds to both the encapsulated strain and the nonencapsulated mutant. The ability of pooled antisera from mice immunized with rNspA to bind to bacteria is also shown. The antisera from mice immunized with wild-type rNspA show an increase in fluorescence intensity with both the encapsulated and nonencapsulated mutant strains when the antisera were tested at dilutions of 1:10 to 1:100. In contrast, polyclonal antisera prepared to proteins precipitated from culture supernatant of the *E. coli* vector alone (without the *nspA*

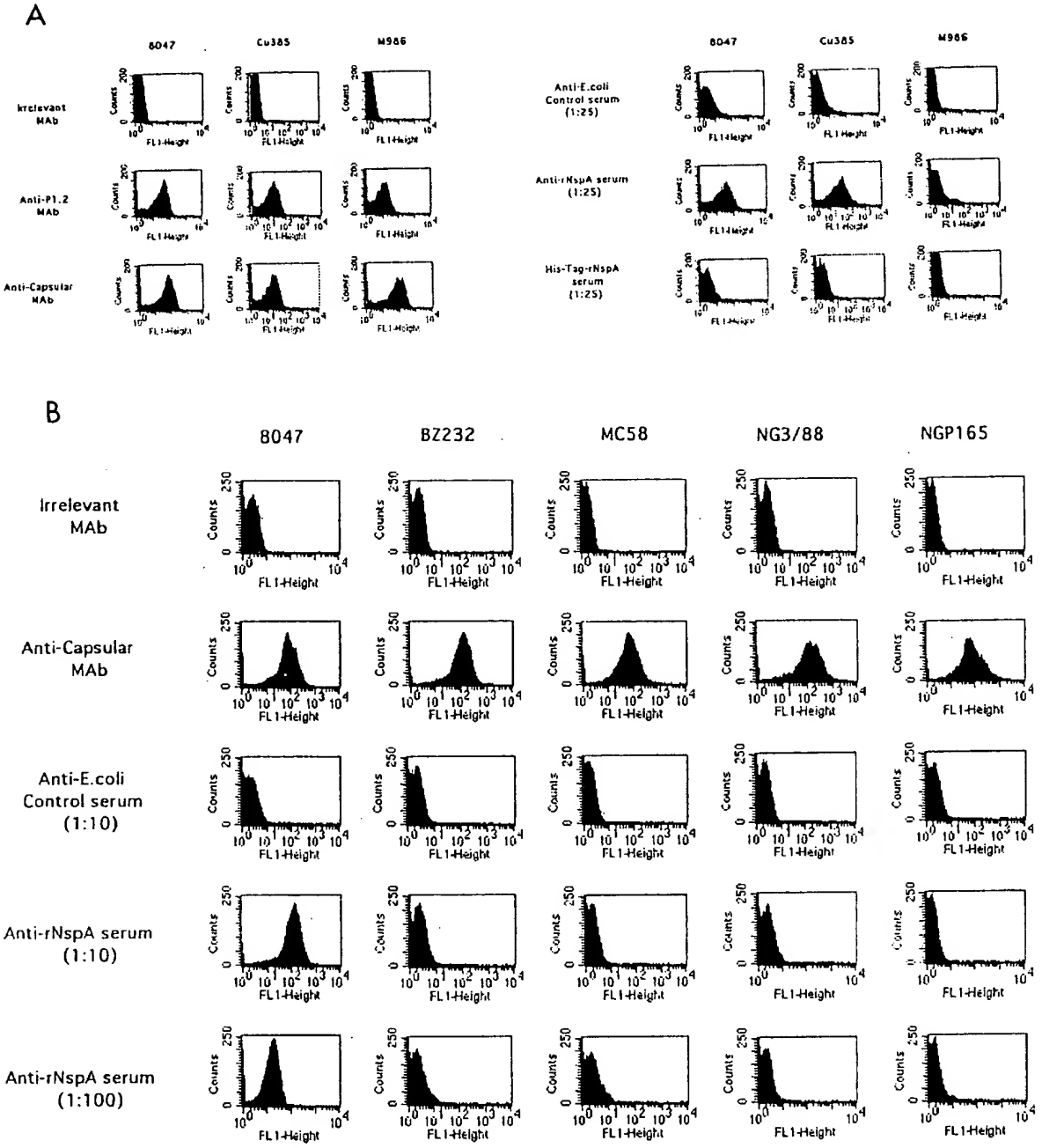

FIG. 3. (A) Binding of polyclonal anti-rNspA antiserum and control MAbs to encapsulated meningococcal B strains 8047, CU385, and M986, as determined by indirect-fluorescence flow cytometry. All sera were tested at a dilution of 1:25. The test and control MAbs and antisera are those described in the legend to Fig. 2. (B) Binding of polyclonal anti-rNspA antiserum and control MAbs to encapsulated meningococcal B strains 8047, BZ232, MC58, NG3/88, and NGP165, as determined by indirect-fluorescence flow cytometry. All sera were tested at dilutions of 1:10 and 1:100 as indicated. The irrelevant and anticapsular control MAbs and control anti-*E. coli* antisera are those described in the legend to Fig. 2.

gene) showed only low-intensity background fluorescence and were considered negative. Note that in Fig. 2, representative data are shown only for antisera diluted 1:25.

As shown in Fig. 2, the antiserum prepared against purified, denatured HisTag-NspA gave background levels of fluorescence with strains NMB and M7 and were considered negative. This antiserum was also negative for bacterial binding with strains 8047, CU385, and M986 (Fig. 3A, right). However, by Western blotting (Fig. 1B), the anti-HisTag-NspA antiserum recognized purified HisTag-NspA (Fig. 1B, lane 1), wild-type rNspA (lanes 3 and 9), as well as NspA present in a detergent-insoluble outer membrane fraction from four different *N. meningitidis* group B strains, including strains 8047, CU385, and M986 (lanes 4 and 8). Taken together, the data suggest that the antibodies prepared to HisTag-NspA recognize epitopes that are present in denatured NspA but not in native NspA present on the surface of live meningococcal B bacteria. In contrast, the antibodies prepared against wild-type rNspA, which were

detected by the flow cytometric binding assay, are likely to be specific for conformational NspA epitopes.

Strain differences in NspA surface accessibility. The flow cytometric assay was used to assess the ability of pooled IgG anti-rNspA antibodies to bind to the surface of 17 genetically diverse strains of live, encapsulated pathogenic *N. meningitidis* group B bacteria isolated from patients residing in different countries over a period of more than 30 years. Figure 3A shows examples of antibody binding to the surface of *N. meningitidis* 8047, the homologous strain from which we cloned the *nspA* gene, and two heterologous strains, CU385 and M986. All three strains showed no binding with a negative control MAb of irrelevant specificity but bound strongly to two positive control MAbs, an anticapsular antibody and anti-P1.2 (left). In contrast, the anti-rNspA antibody bound only to strains CU385 and 8047 and there was no detectable anti-rNspA antibody binding to strain M986 (right).

Figure 3B shows examples of four additional strains, BZ232, MC58, NG3/88, and NGP165, that showed no detectable anti-rNspA antibody binding to the cell surface when tested by flow cytometry at an antiserum dilution of 1:10. By comparison, strain 8047 was strongly positive when tested at dilutions of 1:10 and 1:100. All five strains also showed strong binding to the control anticapsular MAb.

As summarized in Table 1, of the 17 *N. meningitidis* group B strains tested by flow cytometry, 11 (65%) were positive for NspA cell surface binding and the remaining 6 were negative. There was no apparent relationship between cell surface NspA expression in a given strain and the respective serotype or subtype, or electrophoretic type (ET) classification, or the year or country of isolation.

Sequence variability in NspA from different strains. One possible explanation for apparent strain differences in reactivity with the anti-rNspA antisera in the flow cytometric assay is polymorphisms in the NspA protein. To test this possibility, we cloned and sequenced the *nspA* gene from five of the six negative strains (BZ232, NG3/88, NGP165, M136, and M986) and from three of the positive strains (8047, CU385, and NG6/88). The GenBank accession numbers for the *nspA* gene sequences for these strains are AF175676 to AF175683. For the sixth negative strain, MC58, we obtained the nucleotide sequence of the *nspA* gene from data generated by The Institute of Genomic Research, as part of their MC58 genome sequencing studies (60). The *nspA* gene sequences of all 10 strains were highly conserved. In comparison to the DNA sequence published by Martin et al. (36), there were between zero and five nucleotide differences resulting in zero to three amino acid differences. Further, as shown in Fig. 4, with one exception, all of the amino acid variants involved the same respective residues in discrete segments of the protein. These included the signal peptide, which is not present in the mature protein, and two short segments in the carboxyl-terminal 50 amino acids of the protein. Evidently, these differences are inconsequential for recognition of the protein by the anti-rNspA antisera, since there are examples of identical derived amino acid sequences of the mature NspA from strains that were negative for reactivity with anti-rNspA antisera and those that were positive (compare M136 to 8047, NGP165 to NG6/88, and MC58 to CU385).

Detergent-insoluble outer membrane fractions prepared from different *N. meningitidis* group B strains. The indirect-fluorescence cell binding assay provides information on the surface accessibility of NspA epitopes that are likely to be important in interacting with protective antibody. Given the identical or nearly identical *nspA* genes in the different strains, the failure to detect NspA surface expression in some strains but not others could reflect decreased amounts of NspA in the outer membrane of the former, possibly as a result of strain differences in *nspA* gene expression.

To investigate possible strain differences in NspA expression in the outer membrane, we extracted bacterial cell pellets with sodium lauroyl sarcosinate and analyzed the insoluble outer membrane fraction by SDS-PAGE and Western blotting. Data from five representative strains, three that were positive (8047, CU385, and NG6/88) and two that were negative (M986 and M136) for anti-rNspA antisera binding by the flow cytometric assay, are shown in Fig. 1. As described above, a band with an apparent molecular mass of 18.6 kDa was observed in membrane fractions from all five strains analyzed by SDS-PAGE (Fig. 1A), and this band was cross-reactive with anti-HisTag-NspA serum by Western blotting (Fig. 1B). In other experiments (results not shown), NspA was detected in sodium lauroyl sarcosinate-insoluble outer membrane fractions prepared from the remaining four strains that were negative for cell surface binding by the flow cytometric assay (strains NG3/88, MC58, NGP165, and BZ232). NspA was also detected by Western blotting with antisera prepared to partially purified rNspA without the His tag (data not shown).

Among the strains tested, the density of the 18.6-kDa NspA band present in the OMP preparation from strain 8047 (Fig. 1A) and BZ198 (data not shown) appeared to be greater than that of the corresponding bands from the other strains. These observations were consistent in multiple membrane protein preparations from the strains.

Production of capsular polysaccharide. The ability of anti-rNspA antibody to bind to the bacterial cell surface could also be influenced by the amount of polysaccharide capsule present. To test this hypothesis, we compared the quantity of capsular polysaccharide produced by each of the strains that were scored as positive or negative for anti-NspA binding by the flow cytometric assay. As described in Materials and Methods, the bacterial cells were suspended in buffer and incubated with EDTA to release capsular polysaccharide. The amount of soluble capsule released was measured by an inhibition ELISA. The data were expressed as the reciprocal dilution of supernatant giving 50% inhibition of binding of an anticapsular MAb to biotinylated meningococcal B polysaccharide adhering to avidin-coated wells. Each strain was tested for capsular polysaccharide production in two to four independent experiments. From these results, a mean value ($\pm$ standard error) was assigned to each strain. The results for the individual strains are summarized in Table 1. Although there are a few notable outliers (e.g., strains 8047 and NGP165), on average the 6 strains that were negative for reactivity with anti-rNspA antisera by the flow cytometric assay produced threefold more capsular polysaccharide than did the 11 strains that were positive (reciprocal geometric mean dilutions of 676 and 224, respectively [$P < 0.05$ by the t test]). We also measured the amount of capsular polysaccharide released into the culture medium for representative NspA-positive (CU385 and H44/76) and -negative (M136 and M986) strains by the same inhibition ELISA. In each case, the relative amount of polysaccharide released by each strain into the medium correlated directly with the amount of capsule polysaccharide measured in the bacterial extracts (data not shown). Thus, the smaller amounts of cell-associated capsule in the NspA cell surface positive strains were not a result of greater shedding of capsular material into the broth.

Complement-mediated bactericidal activity of anti-rNspA antiserum. Table 1 summarizes the results of measurement of complement-mediated bactericidal activity of the anti-rNspA antiserum for each of the strains tested. All 17 strains were

```
                Signal Peptide                          Loop 1
                  -10          1         10         20         30         40
NM 608 B      MKKALATLIA LALPAAALAE GASGFYVQAD AAHAKASSSL GSAKGFSPRI SAGYRINDL
NM NG6/88+    ---------- ---------- ---------- ---------- ---------- ---------
NM CU385+     ---------- ---------- ---------- ---------- ---------- ---------
NM 8047+      ------A--- ---------- ---------- ---------- ---------- ---------
NM NGP165-    ---------- ---------- ---------- ---------- ---------- ---------
NM MC58-      ---------- ---------- ---------- ---------- ---------- ---------
NM M986-      ---------- ---------- ---------- ---------- ---------- ---------
NM BZ232-     ---------- --I------- ---------- ---------- ---------- ---------
NM M136-      ---------- --I------- ---------- ---------- ---------- ---------
NM NG3/88-    ---------- --I------- ---------- ---------- ---------- ---------
NG FA1090     ------A--- ---------- ---------- --------*- ---------- ---------
NG B2         ------A--- ---------- ---------- ---------- ---------- ---------

                          Loop 2                                       Loop 3
                   50         60         70         80         90        100
NM 608B       RFAVDYTRYK NYKAPSTDFK LYSIGASAIY DFDTQSPVKP YLGARLSLNR ASVDLGGSDS
NM NG6/88+    ---------- ---------- ---------- ---------- ---------- ----------
NM CU385+     ---------- ---------- ---------- ---------- ---------- ----------
NM 8047+      ---------- ---------- ---------- ---------- ---------- ----------
NM NGP165-    ---------- ---------- ---------- ---------- ---------- ----------
NM MC58-      ---------- ---------- ---------- ---------- ---------- ----------
NM M986-      ---------- ---------- ---------- ---------- ---------- ----------
NM BZ232-     ---------- ---------- ---------- ---------- ---------- ----------
NM M136-      ---------- ---------- ---------- ---------- ---------- ----------
NM NG3/88-    ---------- ---------- ---------- ---------- ---------- ----------
NG FA1090     ---------- ---Q------ --------V- ---------- --F------- ---AH-----
NG B2         ---------- ---------- --------V- ---------- --F------- ---AH-----

                                                   Loop 4
                  110        120        130        140        150
NM 608B       FSQTSIGLGV LTGVSYAVTP NVDLDAGYRY NYIGKVNTVK NVRSGELSVG VRVKF
NM NG6/88+    ---------- ---------- ---------- ---------- ---------- -----
NM 8047+      ------T--- --A------- ---------- ---------- ---------- -----
NM CU385+     ---------- ---------- ---------- ---------- ---------A -----
NM NGP165-    ---------- ---------- ---------- ---------- ---------- -----
NM MC58-      ---------- ---------- ---------- ---------- ---------A -----
NM M986-      ---------- ---------- ---------- ---------- ---------- -----
NM BZ232-     ------T--- ---------- ---------- ---------- ---------A -----
NM M136-      ------T--- --A------- ---------- ---------- ---------- -----
NM NG3/88-    ------T--- --A------- ---------- ---------- ---------A -----
NG FA1090     ---K--A--- --A------- ---------- ---V------ ---------A -----
NG B2         ---K--A--- --A------- ---------- ---V------ ---------A -----
```

FIG. 4. Predicted amino acid sequences of NspA from different *N. meningitidis* serogroup B strains. Sequencing data for *N. meningitidis* 608B were obtained from Martin et al. (36) (GenBank accession no. U52066), and those for strain MC58 were obtained from The Institute for Genomic Research (49a). Data for *N. gonorrhoeae* B2 were from GenBank (accession no. U52069), and data for FA1090 were from the University of Oklahoma *Neisseria gonorrhoeae* Genome Sequencing Project. The numbering corresponds to that of the mature protein. Underlined segments are putative surface-exposed loops (see Fig. 5). NM indicates that the NspA sequence is from *N. meningitidis*, and NG indicates that it is from *N. gonorrhoeae*. The superscript + and − indicate strains that were positive or negative, respectively, for anti-rNspA antiserum binding as determined by the flow cytometric assay.

killed by complement together with similar concentrations of a positive control anti-capsular MAb (SEAM 12; subtype IgG2a [21]). In contrast, the six strains that were negative for anti-rNspA antiserum binding by the flow cytometric assay were resistant to anti-rNspA antibody-induced complement-mediated bactericidal or bacteristatic activity (each showed a 150 to 200% increase in CFU per milliliter compared to the control CFU per milliliter at time zero when assayed at the lowest dilution of the anti-NspA antiserum tested, 1:4).

Of the 11 strains that were positive for NspA surface expression by flow cytometry, 8 (73%) (SWZ107, J1H5341, CU385, NG6/88, BZ198, H44/76, NMB, and 8047) were killed by complement and the anti-rNspA antiserum and 3 were not killed (strains 1000, H355, and S3446). Of interest, with the exception of strain 1000, growth of the other two NspA cell surface-positive strains that were not killed in the assay was inhibited by the anti-rNspA antiserum and complement (survival after a 60-min incubation was less than 100% but greater than 50%,

TABLE 2. Protective activity of anti-rNspA antisera[a]

Treatment	Dose/rat or serum dilution	Strain	Blood culture		
			No. positive/ total no.	10^3 mean CFU/ml	CFU/ml (% of control)[b]
Anticapsular MAb	2 μg	M986	0/6	<1	<1
Anti-rNspA	1:5	M986	6/6	44[c]	45
Anti-rNspA	1:25	M986	6/6	93[c]	95
Anti-*E. coli* negative control	1:5	M986	6/6	98[c]	
Anticapsular MAb	2 μg	8047	0/5	<1	<1
Anti-rNspA	1:5	8047	1/6	0.2[d]	2
Anti-rNspA	1:25	8047	1/5	0.4[d]	4
Anti-*E. coli* negative control	1:5	8047	6/6	10[d]	

[a] Infant rats (6 days old) were challenged i.p. with 100 μl of buffer containing 5.4×10^3 CFU of strain M986 (negative for NspA surface-accessible epitopes) or 6.3×10^3 CFU of strain 8047 (positive for NspA surface-accessible epitopes) together with antiserum or MAb. Quantitative blood cultures were obtained 18 h later. For calculation of geometric mean CFU per milliliter, animals with sterile cultures were assigned values of 0.1 CFU/ml.

[b] Calculated in comparison to geometric mean CFU per milliliter for animals challenged with the respective strain.

[c] $P > 0.5$ compared to geometric mean CFU per milliliter for respective control rats.

[d] $P < 0.001$ compared to geometric mean CFU per milliliter for respective control rats.

compared to the CFU per milliliter at time zero). This inhibition was complement dependent (not detected with heat-inactivated complement) and, as noted above, was not observed in the six strains that were NspA cell surface negative.

Passive protection by anti-rNspA antiserum. The ability of the anti-rNspA antiserum to confer passive protection against bacteremia was evaluated in infant rats subjected to i.p. challenge. Two representative strains were selected for this study, i.e., M986, which was negative for NspA surface accessibility by the flow cytometric assay and resistant to anti-NspA-induced bactericidal activity, and 8047, which was positive for NspA surface-accessible epitopes and susceptible to anti-NspA bactericidal activity. The challenge doses used were similar for the two strains (5.5×10^3 cells of strain M986 and 6.3×10^3 cells of 8047). To maximize the likelihood of observing protection, the bacteria were resuspended in buffer and different dilutions of test or control antisera immediately before the challenge. Table 2 summarizes the results of quantitative bacterial cultures performed on blood specimens obtained 18 h after challenge. A dose of 2 μg per rat of the positive control anticapsular MAb, SEAM 3, was completely protective against both strains. Two dilutions (1:5 and 1:25) of the anti-rNspA antiserum (the highest dilutions tested) protected against bacteremia caused by strain 8047 (positive for NspA surface epitopes). However, neither dilution conferred protection against strain M986 (negative for NspA surface-accessible epitopes).

DISCUSSION

In 1997, Martin et al. described NspA as a potential vaccine candidate for the prevention of invasive *N. meningitidis* disease (36). NspA epitopes appeared to be highly conserved across *N. meningitidis* based on reactivity with a murine anti-NspA MAb, designated Me-1. This MAb cross-reacted with 248 of 250 meningococcal strains in a colony blot ELISA with whole bacterial antigens. This strain collection included representatives of serogroups A, B, C, 29E, W-135, Y, and Z (including 44 serogroup B organisms). The MAb also was bactericidal against isolates with serogroups A, B, or C. In mice, rNspA administered with QuilA elicited serum bactericidal antibody responses against representative strains of serogroups A, B, and C and protected animals from challenge with live *N. meningitidis* group B bacteria. Recently, rNspA absorbed to alum also was reported to elicit serum meningococcal bactericidal antibody responses in rabbits and monkeys (37). Based on these data, an rNspA vaccine is being developed for the prevention of meningococcal disease caused by all serogroups. However, serogroup A and C polysaccharide-protein conjugate vaccines are highly immunogenic in human infants (8, 16, 29, 32) and also induce polysaccharide-specific memory B cells (28, 30). Thus, the greatest utility for an rNspA vaccine would be for prevention of serogroup B disease, since, to date, no other approach to vaccination against serogroup B strains has proven to be safe and broadly effective (18). Therefore, in the present study, we focused on further characterization of NspA in a collection of 17 *N. meningitidis* group B strains that were selected based on ET and/or multilocus sequence typing to be genetically highly diverse (Table 1).

NspA is reported to be accessible at the surface of all intact *N. meningitidis* strains tested (36, 44). However, we found no detectable anti-rNspA antibody binding to the bacterial surface of 6 (35%) of 17 meningococcal B strains as assessed by indirect-fluorescence flow cytometry. The six strains that were negative for anti-rNspA antiserum binding had *nspA* genes that were identical or nearly identical to those of representative strains that were positive for anti-rNspA cell surface binding. Furthermore, based on SDS-PAGE and Western blotting, all six strains that were negative for anti-rNspA antisera binding contained NspA in detergent-insoluble outer membrane preparations. Thus, failure of these strains to express NspA epitopes on the bacterial surface is not a result of absence of the gene, lack of expression of the gene, or polymorphisms.

At present it is unclear why there are strain differences in NspA surface epitopes. One possible clue is our finding that the 6 strains that were negative for surface-reactive NspA produced, on average, greater than threefold more capsular polysaccharide than did the 11 strains that were positive for surface-reactive NspA. Conceivably, the presence of larger amounts of capsule interferes with the ability of the anti-rNspA antibody to bind to NspA epitopes, which, in strains with smaller amounts of capsule, are accessible to antibodies. To gain insight into the possible antigenic sites of NspA and into why NspA surface accessibility might be restricted by the presence of increased amounts of capsular polysaccharide, we have developed a tentative model for the structure and the organization of NspA in the bacterial membrane (Fig. 5).

Based on the pattern of alternating hydrophobic and hydrophilic amino acids, which is characteristic of many β-barrel porin structures (66), we predict that NspA contains eight transmembrane β-strands and four surface-exposed connecting loops. According to Martin et al., the only significant homology between the deduced amino acid sequence of NspA and those of other proteins are weak homologies to the *Neisseria* opacity protein (Opa) family in two small segments (~20 amino acids) near the C-terminal end of the protein (36). However, when we compared the N-terminal and C-terminal halves of NspA separately with sequences in the nonredundant GenBank CDS database (1), we found a high degree of homology (>40% identity and >70% similarity) between NspA and Opa proteins from *N. meningitidis*, *N. gonorrhoeae*, *N. flavius*, *N. sicca*, and *H. influenzae* (data not shown). The Opa proteins are thought to be integral membrane proteins that have eight transmembrane segments and a β-barrel topology in

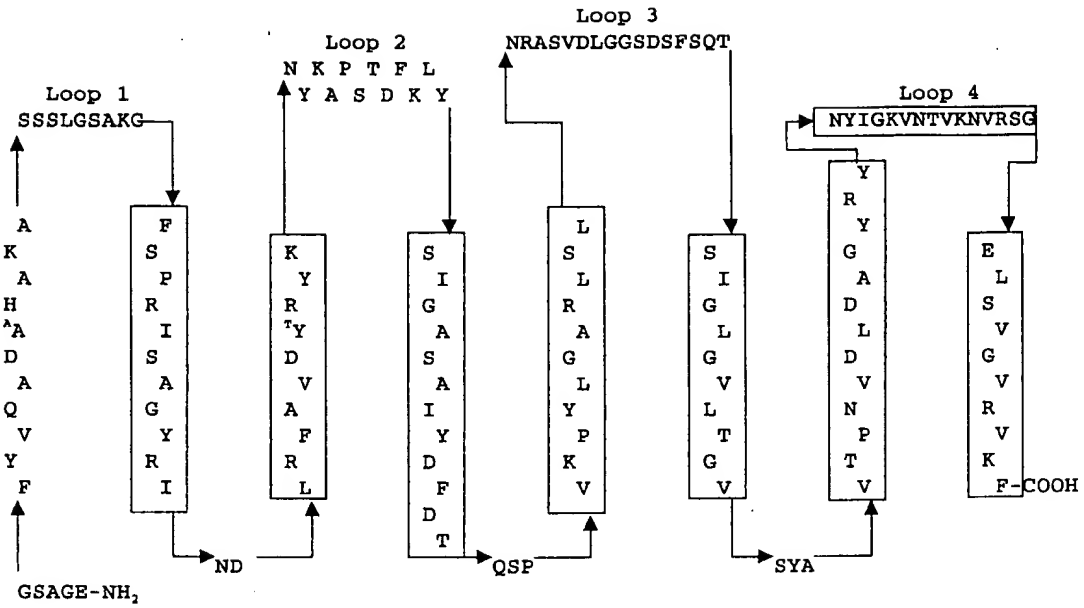

FIG. 5. Secondary-structure prediction for NspA. The boxed areas indicate segments that are >40% identical and >70% similar to encoded amino acid sequences of opacity proteins (Opa) from *N. meningitidis*, *N. gonorrhoeae*, *N. flavius*, *N. sicca*, and *H. influenzae* identified in a BLAST sequence alignment search (1) of the Non-Redundant GenBank CDS. The segments having alternating placement of letters are predicted amphiphilic β-strands. Vertical segments correspond to putative transmembrane segments. Segments at the top of the figure are predicted to be surface-exposed loops, which are labeled loops 1 to 4.

the membrane similar to that of porin (34). The presence of NspA in detergent-insoluble membrane preparations indicates that NspA is located in the outer membrane of meningococci, which would be consistent with the Opa-like membrane topology shown in the model. In addition, the segments of NspA that are most similar to those of the Opa proteins are the putative transmembrane segments and loop 4 indicated by the boxed areas in Fig. 5. Finally, as was found for Opa (46), NspA is a heat-modifiable protein, as shown in Fig. 1C.

The opacity proteins of *Neisseria* may be virulence factors (65) and, under certain circumstances, can elicit protective antibody (50). However, problems with limited antibody accessibility of the opacity proteins in encapsulated bacteria, variability of amino acid sequences in exposed loop segments, and phase variation of protein expression during clinical infection have limited the ability of Opa to elicit protective antibody consistently (34).

In contrast, there appears to be little or no sequence variation in the putative surface-exposed loops of NspA based on the sequence data produced in this study and by others. However, Plante et al. recently reported that a panel of anti-*N. meningitidis* NspA MAbs that reacted with all meningococcal strains tested, reacted with only a limited number of *N. gonorrhoeae* strains, even though the respective amino acid sequences in the two species are 92% identical (44). When the respective NspA sequences of the meningococcal and gonococcal strains were compared (Fig. 4), all of the respective amino acid differences that resulted in changes in hydrophobicity or charge were found to be located in the putative surface-exposed connecting loops (Fig. 5). This finding suggests that the connecting loops in NspA, which are highly conserved in *N. meningitidis*, may be important epitopes for antibodies that bind to native meningococcal NspA. However, the putative surface loops of NspA are relatively small (10 to 16 amino acids) compared to, for example, the highly immunogenic external VR1 and VR2 loops (loop 1 and loop 4, respectively) of PorA (~10 to 40 amino acids) (61). Although there is some overlap, the NspA loops would appear to be smaller, which might account for lower surface accessibility of NspA epitopes, especially in the presence of abundant capsular polysaccharide.

The full implications of our data with respect to the potential protective efficacy of an rNspA vaccine require additional study. However, our observations suggest that approximately one-third of meningococcal B strains have decreased expression of cell surface NspA epitopes when grown in vitro. Furthermore, strains that are negative for NspA cell surface epitopes are also resistant to anti-NspA-induced complement-mediated bacteriolysis, and for one of the strains tested in the infant-rat model, the anti-NspA antiserum failed to confer protection against bacteremia. In contrast, this antiserum was highly protective against a test strain that was positive for NspA surface-accessible epitopes and that was susceptible to anti-NspA antibody-induced bactericidal activity. Finally, the anti-rNspA-resistant strains also tended to be the same strains that produced the largest amounts of capsular polysaccharide and therefore would be expected to have the greatest virulence. Taken together, these data raise concerns about the ability of a vaccine containing only rNspA to confer broadly protective immunity against meningococcal B disease. An rNspA vaccine, therefore, may need to be supplemented by the inclusion of additional antigens, such as PorA, which is an important target of bactericidal antibody in humans (18). However, before reaching this conclusion, it will be important to investigate the immunogenicity of NspA in humans and to determine whether the addition of other candidate meningococcal antigens to an rNspA vaccine can augment protective immune responses.

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